Immunological and epidemiological investigations in South African ostriches and penguins

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Declaration

I, the undersigned, herby declare that the work contained in this dissertation is my own original work and that I have not previously in its entirety or in part submitted it at any university for a degree.

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Opsomming

Newcastlesiekte (NS) en mikoplasmainfeksies in voltruise het geweldige ekonomiese implikasies vir die Suid-Afrikaanse volstruisbedryf. Die rede hiervoor is dat NS 'n beperkende faktor in die uitvoer van volstruisprodukte na die Europese Unie is, en mikoplasmainfeksies tot kudde verliese, verlaagde produksie en uitbroei asook lae gradering van karkasse lei. In die eerste gedeelte van hierdie proefskrif is die rol van passiewe- en mukosale-immuniteit in die beskerming van volstruiskuikens teen NS virus (NSV) ondersoek. Volstruishenserum IgG en eier IgY is geïsoleer en gekarakteriseer en die oordrag van maternale anti-NSV antiliggame na die eier ondersoek met behulp van 'n 'enzyme-linked immunosorbent assay' (ELISA). Resultate het getoon dat anti-NSV antiliggame suksesvol van die hen na die eier oorgedra is. Volstruis IgA is ook geïsoleer, gekarateriseer en konyn anti-volstruis IgA antiliggame geproduseer wat gebruik is vir die bepaling van mukosale anti-NSV IgA antiliggame in reaksie op mukosale immunisering. Resultate het getoon dat lewendige La Sota entstof IgA produksie stimuleer en dus tot mukosale-immuniteit in volstruiskuikens lei. In die tweede gedeelte van hierdie proefskrif is volstruismikoplasmas geïsoleer en geïdentifiseer met behulp van 16S rRNA geenopeenvolgingsbepalings. Hierdie volgordes het getoon dat drie unieke mikoplasmas in volstruise voorkom wat filogeneties verskillend blyk te wees. Die 16S rRNA geenopeenvolgings van die volstruismikoplasmas is gebruik vir die ontwikkeling van spesifieke inleiers vir die PKR identifisering en diagnose van mikoplasmainfeksies in volstruise.

Die laaste gedeelte van hierdie proefskrif fokus op voëlmalaria in die Afrika pikkewyn en die bestuur van hierdie siekte gedurende rehabilitasie. Die 'South African Foundation for the Conservation of Coastal Birds' (SANCCOB) is 'n seevoëlreddings- en rehabilitasie-sentrum vir siek, beseerde en ge-oliede pikkewyne. Hierdie sentrum het egter aansienlike vrektes as gevolg van voëlmalaria. In hierdie studie is 'n ELISA ontwikkel vir die bepaling van natuurlike anti-*Plasmodium* antiliggaamvlakke van pikkewyne by aankoms en tydens rehabilitasie by SANCCOB. Resultate het 'n toename in anti-*Plasmodium* antiliggaamvlakke getoon na toelating wat nie beïnvloed is deur olie nie. Hierdie toename kan toegeskryf word aan nuwe malariainfeksies en nie 'n heruitbraak van bestaande infeksies nie wat daarop dui dat pikkewyne aan voëlmalaria blootgestel word by die SANCCOB-sentrum.

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Summary

Newcastle disease (ND) and mycoplasma infections in ostriches have considerable economic implications for the South African ostrich industry in that ND is a limiting factor in the export of ostrich products to the European Union and mycoplasma infections cause stock losses, reduced production, reduced hatchability and downgrading of carcasses. In the first section of this dissertation, the role of passively acquired and mucosal immunity in protection of ostrich chicks against Newcastle disease virus (NDV) was investigated. Ostrich hen serum IgG and yolk IgY were isolated and characterized, and the transfer of maternal anti-NDV antibodies to the egg yolk was determined using an enzyme-linked immunosorbent assay (ELISA). Results indicated that anti-NDV antibodies were successfully transferred from the ostrich hen to the egg yolk. In addition, ostrich IgA was isolated, characterized and rabbit anti-ostrich IgA antibodies produced and used for measuring mucosal anti-NDV IgA antibodies produced in response to mucosal vaccination. Results indicated that the live La Sota vaccine stimulates IgA production and thus mucosal immunity in ostrich chicks. In the second section of this dissertation, ostrich mycoplasmas were isolated and identified using 16S rRNA gene sequencing. These sequences indicated that ostriches carry three unique mycoplasmas, which are phylogenetically quite divergent. The 16S rRNA gene sequences of the ostrich mycoplasmas were subsequently used for the development of specific primers for the detection and diagnosis of mycoplasma infections in ostriches by PCR.

The last section of this dissertation focuses on avian malaria in African penguins and the management of this disease during rehabilitation. The Foundation for the Conservation of Coastal Birds (SANCCOB) is a seabird rescue and rehabilitation centre, which is largely dedicated to the rehabilitation of diseased, injured and oiled penguins. Significant mortalities due to avian malaria occur at this facility. The aim of this study was the development of an ELISA for the purpose of assessing the natural levels of anti-*Plasmodium* antibodies in African penguins on entry into the SANCCOB facility and during rehabilitation. Results indicated significant increases in anti-*Plasmodium* antibody levels after entry, which was not influenced by oiling. Infection with malaria and not parasite recrudescence was viewed to be the cause of this increase, indicating a possible role of the SANCCOB facility in exposing penguins to avian malaria.

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Abbreviations

ABTS	2,2'-Azino-bis[3-ethylbenzthiazoline-6-sulfonic acid
Ala	Alanine
Anon	Anonymous
Asn	Asparagine
Asp	Aspartic acid
bp	Base pair
BSA	Bovine serum albumin
Cbis	Concentration N,N'-Methylene-bis-acrylamide
CTAB	N-cetyl-N,N,N-trimethyl ammonium bromide
CS	Circumsporozoite
Da	Dalton
DAPSA	DNA and Protein Sequence Alignment
DMF	N,N-dimethylformamide
DMSO	Dimethylsulfoxide
dNTP	Deoxyribonucleotide triphosphates
EDTA	Ethylene diamine tetra-acetic acid di-sodium salt
ELISA	Enzyme-linked immunosorbent assay
FAT	Fluorescent Antibody Test
GLM	General Linear Models
h	Hour
HEPES	(N-[2-Hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid])
HG	Harderian gland
HI	Haemagglutination inhibition
Ig	Immunoglobulin
IOE	Inactivated oil emulsion
IUCN	The World Conservation Union
kbp	Kilo base pair
kDa	Kilodalton
LSD	Least significant difference
mA	Milliampere
mAb	Monoclonal antibodies
min	Minutes
Mr	Relative molecular weight

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NB	Naked bacteria
ND	Newcastle disease
NDV	Newcastle disease virus
NEM	N-ethylmaleimide
NVND	Neurotropic Velogenic Newcastle Disease
P	Probability
PAP	Peroxidase anti-peroxidase
PAUP	Phylogeny analysis using parsimony
PEG-Alc	Polyethylene glycol and alcohol
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
Pro	Proline
RB	Reaction buffer
Rf	Relative mobility
RNA	Ribonucleic acid
rRNA	Ribosomal Ribonucleic acid
RT	Room temperature
S	Svedberg unit
SAMSA	S-acetylmercaptosuccinic anhydride
SANCCOB	South African Foundation for the Conservation of Coastal Birds
SAS	Statistical Analysis System
SC	Secretory component
SDS-PAGE	Sodium dodecyl sulphate gel electrophoresis
sec	Seconds
Sulfo-SMCC	Sulfosuccinimidyl 4-[N-maleimidomethyl]-cyclohexane-1-carboxylate
T	Total concentration of Acrylamide and N,N'-Methylene-bis-acrylamide
TAE	Tris-base, glacial acetic acid, EDTA
TE	Tris-EDTA
Temed	N,N,N',N'-tetramethylethylendiamine
T_m	Melting temperature
UPGMA	Unweighted pairgroup method with arithmetic mean
UV	Ultra-violet
Val	Valine
VN	Virus neutralization
v/v	Volume per volume
VVND	Viscerotropic Velogenic Newcastle Disease
w/v	Weight per volume

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CHAPTER 1:
Introduction

The research, on which this dissertation is based, aims to address specific problems concerning the immunology and epidemiology of diseases within South African ostriches (*Struthio camelus*) and penguins (*Spheniscus demersus*). Although these birds and their diseases are distinctly different, the research methodologies applied are shared and therefore form a meaningful study with a common theme.

The ostrich research was industry driven and focused on Newcastle disease (ND) and mycoplasma infections in ostriches, both having economic implications for the ostrich industry. The research in African penguins was aimed towards conservation and focused on avian malaria and the management of this disease during rehabilitation of penguins.

1.1. NEWCASTLE DISEASE AND MYCOPLASMA INFECTIONS IN OSTRICHES

This research was funded by the Klein Karoo Co-operative, which is the foremost supplier of ostrich products, supplying about 70% of all ostrich products on international markets. In 2002, the annual income of the Klein Karoo Co-operative for all ostrich-related products totalled about R500 million, with ostrich meat specifically, providing an income of R200 million.

1.1.1. NEWCASTLE DISEASE

An increase in commercial ostrich production and subsequent international trade has drawn attention to the significance of highly contagious diseases such as ND. ND has had a major impact on poultry health on a global scale with resulting limits being placed on the international trade of poultry and poultry products (Swayne and King, 2003). In South Africa this has had specific repercussions for the ostrich industry, as ND is a limiting factor in the export of ostrich products and especially ostrich meat to the European Union, due to the possible transmission of Newcastle disease virus (NDV) to poultry. This has prompted immunological investigations in ostriches, which have been conducted by the Department of Biochemistry at the University of Stellenbosch and the Klein Karoo Co-operative in Oudtshoorn since 1996.

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Vaccination trials were undertaken to assist the industry in establishing optimal vaccination schedules for slaughter ostriches against NDV. This research was conducted as part of the author's M.Sc. Thesis (Blignaut, 1998) and culminated with the publication of these results in Avian Diseases (Blignaut *et al.*, 2001). This research, along with studies done by Verwoerd *et al.* (1999) on the experimental infection of vaccinated slaughter ostriches, assisted the ostrich industry in establishing optimal levels of immunity to ensure that ostriches would not transmit NDV via their meat. This had important economic implications in that permission for the continued export of ostrich meat to the European Union was granted on the basis of the results of this research. However, results obtained during the vaccination trials indicated that the current vaccination regimes used in ostrich chicks are suboptimal in producing immunity to NDV.

The aim of the current study was therefore to evaluate the maternal and mucosal immune mechanisms involved in NDV immunity in ostrich chicks with the future goal of establishing effective vaccination schedules for ostrich chicks against NDV. A literature overview of different epidemiological and immunological aspects of ND is given in Chapter 2. Although the main focus of this chapter is on ostriches, limited research on ND in ostriches required some aspects of ND to be discussed in relation to poultry. The results on the characterization of maternal and mucosal immunity in ostrich chicks obtained in this study are presented in Chapter 3 as an independent manuscript, to facilitate publication.

1.1.2. MYCOPLASMA INFECTIONS

An increase in commercial ostrich production has also lead to a system of intensive rearing, which has brought certain disease syndromes to the fore. Mycoplasma infections have been implicated, either as primary or secondary causes of disease syndromes. Mycoplasma infections have become a major problem in that it causes high mortalities in ostrich chicks and in the case of slaughter ostriches, leads to the downgrading of carcasses and therefore has a significant economic impact on the ostrich meat industry. Poultry mycoplasmas have previously been isolated from ostriches and it was thought that surrounding commercial poultry kept in close proximity might act as source of infection.

In this study, the possibility that ostriches are in fact being infected by ostrich specific mycoplasmas, and not poultry mycoplasmas, was investigated. The aim of this study

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was to identify ostrich mycoplasmas using the 16S rRNA gene and subsequently to establish their phylogenetic relationships to other avian mycoplasmas. Using these 16S rRNA sequences, a polymerase chain reaction (PCR) assay was developed for the specific detection of ostrich mycoplasmas. A literature overview of avian mycoplasmas is given in Chapter 4. Due to the focus of this study being on the identification of mycoplasmas in ostriches, the outline of Chapter 4 is slightly different to that of Chapter 2 in that a more detailed discussion is given on the classification of mycoplasmas. Due to the susceptibility of ostriches to poultry mycoplasmas, poultry mycoplasmas are discussed in detail separate from that of ostriches. The results of this study are presented in the form of an independent manuscript at the end of Chapter 5.

1.2. AVIAN MALARIA IN AFRICAN PENGUINS

Currently "The World Conservation Union" (IUCN) Red List of Threatened Species™ classifies the status of the African penguin as 'Vulnerable' as it has undergone an observed rapid population decline since the beginning of the 20th century. The initial decline in penguin numbers has been as a result of habitat degradation, harvesting of penguin eggs and ongoing human disturbances. Currently, oil pollution is seen as an important immediate threat to African penguin populations (Nel *et al.*, 2003). The South African Foundation for the Conservation of Coastal Birds (SANCCOB), established in 1968, is a seabird rescue and rehabilitation centre located at Tableview in Cape Town and is dedicated largely to the de-oiling and rehabilitation of African penguins. Demographic impact studies indicated that largely due to the de-oiling efforts of SANCCOB, the 2002 population of African penguins is now 19% larger than it would have been without rehabilitation (Ryan, 2003). Despite the success of SANCCOB in rehabilitating penguins, they have significant mortalities due to avian malaria (Brossy, 1992; Parsons, 2001). The possibility of avian malaria being contracted by penguins at the rehabilitation facility causes concern as to the dangers of introducing avian malaria into already declining wild populations (Brossy, 1992; Graczyk *et al.*, 1995a; Hockey, 2001).

The final section of this dissertation deals with the problem of avian malaria at the SANCCOB rehabilitation facility. Knowledge concerning the epidemiology of avian

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malaria in wild African penguin populations is needed in order to effectively manage and control this disease during rehabilitation.

The aim of this study was the development of an enzyme-linked immunosorbent assay (ELISA) for the purpose of assessing the natural levels of anti-*Plasmodium* antibodies in African penguins on entry into the SANCCOB facility and during rehabilitation with a view to increasing survival rates in the facility. An overview of avian malaria, with the specific focus on African penguins is given in Chapter 6. Results are once again presented as an independent manuscript at the end of Chapter 7.

A final summary and future prospectives for each of the three sections is given in Chapter 8. To avoid duplication, literature cited in each of the above manuscripts will be listed along with the rest of the literature references at the end of the dissertation. Following the literature references is an Appendix containing a sequence alignment of the ostrich isolated mycoplasmas as well as the data used for statistical analysis of the results in Chapter 3 and Chapter 7.

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CHAPTER 2:

Newcastle Disease in Ostriches

2.1. NEWCASTLE DISEASE

2.1.1. HISTORY

A worldwide, ever expanding poultry industry along with international trade has drawn attention to the significance of highly contagious diseases such as Newcastle disease (ND) (Alexander, 1988). The first reported outbreak of ND was in 1926 on a poultry farm near Newcastle-upon-Tyne, England and hence the name (Doyle, 1927). Simultaneous outbreaks occurred in 1926 on the island of Java, Indonesia and Korea and a year later (1927) in Ranikhet, India. It is, however, possible that the disease was already present in Korea as early as 1924, but went unnoticed due to the lack of available expertise in recognising the disease (Clubb, 1986; Alexander, 1988; Cross, 1991). Within a few years after the first diagnosis in 1926, ND spread throughout the world impacting on poultry health on a global scale and subsequently limiting international trade in poultry and poultry products (Swayne and King, 2003). In South Africa this has had specific repercussions for the ostrich industry thereby limiting the export of ostriches and ostrich products to specifically the European Union.

2.1.2. CLASSIFICATION AND CHARACTERISTICS

ND is a highly contagious viral disease caused by Newcastle disease virus (NDV), which is classified as follows (Alexander, 1996; Alexander, 1997):

Order: *Mononegavirales*

Family: *Paramyxoviridae*

Members of this family have an enveloped non-segmented, single stranded, negative sense ribonucleic acid (RNA) genome (Della-Porta and Spencer, 1989). Capsid assembly takes place in the cytoplasm of the infected host cell and the envelope or membrane is derived from the host cell plasma membrane (Mori *et al.*, 1994; Alexander, 1996).

Subfamily: *Paramyxovirinae*

This subfamily contains three genera, *Morbillivirus*, *Paramyxovirus* and *Rubulavirus*.

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Genus: *Rubulavirus*

The type species for this genus is mumps virus and NDV is placed in this genus along with the other avian paramyxoviruses. Members of this genus have highly pleomorphic virus particles, being roughly spherical or filamentous (Alexander, 1996). Typical properties of this genus include a herringbone nucleocapsid and the surface of the virus particle is covered with large and small projections or spikes. The genome codes for the following six viral proteins (Alexander, 1997):

i) Large (L)-protein: a transcriptase (Mori *et al.*, 1994).

ii) Nucleocapsid (NP)-protein.

iii) Phosphorylated nucleocapsid-associated (P)-protein.

iv) Matrix (M)-protein.

v) Haemagglutinin-neuraminidase (HN)-glycoprotein.
This protein is associated with the haemagglutination and neuraminidase activity of NDV. The HN-glycoprotein enables the virus to bind to its cellular receptor on the host cell and the enzyme neuraminidase allows virus to break away from the host cell by receptor destruction (Della-Porta and Spencer, 1989; Cosset *et al.*, 1991; Considine, 1993; Mori *et al.*, 1994; Alexander, 1996).

vi) Fusion (F)-glycoprotein.
This protein is associated with the ability of the virus envelope to fuse with the host cell membrane thus allowing insertion of the virus genome into the host cell. This protein is also responsible for fusion and haemolysis of infected cells (Della-Porta and Spencer, 1989; Cosset *et al.*, 1991; Mori *et al.*, 1994; Alexander, 1996).

The L-, NP- and P-proteins are associated with the RNA to form a coiled nucleoprotein complex that is surrounded by a bilayer lipid membrane containing the M-protein and the HN- and F-glycoproteins. These two glycoproteins are partly inserted into the envelope, while the rest of the protein is exposed on the outside surface of the virus particle as large (HN) and small (F) projections or spikes (Della-Porta and Spencer, 1989; Mori *et al.*, 1994).

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The different avian paramyxoviruses can further be divided into nine serogoups (PMV-1 to PMV-9), with NDV being the type species for PMV-1. All isolates or pathotypes within a single group are antigenically similar although they may vary in their virulence, immunogenicity, ability to spread or be transmitted, heat stability and their ability to elute from erythrocytes after attachment via the HN-glycoprotein (Cross, 1991). Some degree of immunological cross-reactivity also exists between certain viruses within different serotypes (Alexander, 1996; King, 1996). The seemingly minor antigenic difference between strains within a serogroup allows cross-protective immunity between strains and therefore a vaccine developed against one strain can provide protection against several other strains (Cosset *et al.*, 1991; Alexander, 1995b).

2.1.3. PATHOGENICITY

The pathogenicity of a specific strain varies between different host species and is firstly and most importantly determined by the strain type, followed by factors such as (Alexander, 1997):

- the dose of infective virus;
- the route of infection, infection via the nasal, oral or ocular route will result in respiratory infections whilst intramuscular, intravenous and intracerebral infection will enhance neurological signs;
- age of the host, generally the younger the bird the more acute the disease with no clinical signs and sudden death, but in older birds the disease may show characteristic clinical signs and be more protracted;
- environmental conditions and
- concurrent infections within the host.

On the basis of clinical manifestation of ND under laboratory conditions, the different pathotypes of NDV are further divided into (Alexander, 1996; Seal *et al.*, 1996):

i) Velogenic NDV, which is further divided into viscerotropic velogenic (VVND) or neurotropic velogenic (NVND) viruses. VVND causes acute lethal infections with haemorrhagic lesion in the intestine, whilst NVND causes

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respiratory and neurological disease followed by high mortality, but gut lesions are absent (Alexander, 1995b).

ii) Mesogenic NDV, which causes respiratory and neurological signs but with low mortality (Alexander, 1995b). Strains that have been used in vaccines include Roakin, Komarov, Herts and Mukteswar (Meulemans, 1988).

iii) Lentogenic NDV, which causes mild infections of the respiratory tract (Alexander, 1995a; Alexander, 1995b). Lentogenic strains that are mostly used in vaccines include La Sota and Hitchner B1 (Meulemans, 1988).

iv) Asymptomatic enteric NDV, which causes avirulent infections and replication is primarily in the gut such as Ulster 2C (Alexander, 1995a; Alexander, 1995b).

Both the HN- and F- glycoproteins have been shown to be immunogenic in immunization studies and are associated with virus virulence (Della-Porta and Spencer, 1989; Morrison *et al.*, 1990; Cosset *et al.*, 1991). The HN-glycoprotein of different NDV strains has different stretches of amino acid sequences that correlate with virulence (Della-Porta and Spencer, 1989). The F-glycoprotein on the other hand is synthesized as a non-functional precursor, F0, which has to be cleaved to F1 and F2 by host proteases in order to be activated. The ability of the virus to replicate in different cell types is influenced by the F-glycoprotein cleavage site sequence, which differs according to strain virulence, as well as the availability of cellular proteases needed to cleave the precursor protein. Enzymes required to cleave virulent strains' F-glycoproteins have a much wider tissue distribution than enzymes cleaving the F-glycoproteins of non-virulent strains and therefore, virulent strains can affect a wider range of tissues in infected birds than low- or avirulent strains (Considine, 1993; Seal *et al.*, 1996).

2.2. EPIDEMIOLOGY

2.2.1. DISTRIBUTION AND SPECIES AFFECTED

It is difficult to determine the precise geographical distribution of ND strains because of the general use of live vaccines in poultry throughout the world. Many countries have been able to successfully eliminate both velogenic and mesogenic NDV, but

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virulent strains of ND are still a serious problem either as an enzootic disease or as a cause of regular epizootics throughout Africa, tropical Asia and Central and South-America. Epizootics in Europe, on the other hand, tend to be more sporadic in nature (Alexander, 1995b; Verwoerd, 2000). In South Africa specifically, ND is an endemic disease, which means that the disease is normally under control, but outbreaks do occur from time to time (Travers, 1994).

ND has been reported to occur throughout the year in some countries, while in others is seems to have a seasonal occurrence whereby outbreaks coincide with specific seasons such as winter or summer with hot, dry or hot and humid conditions, or it is often associated with the change of season. It is, however, possible that ND is not really associated with any particular season, but rather with periods of climatic stress (Awan *et al.*, 1994). Current and future distribution of ND depends on attempts at both control and eradication of this disease and whether or not these attempts succeed, is greatly dependent on the nature of the poultry industry in the relevant country. Usually countries with predominantly large commercial flocks tend to have fewer problems than countries with mostly village chicken flocks (Alexander, 1997).

Despite the first emergence of ND being in fowl, and the fact that the chicken is regarded as the major host of this disease, all avian species are considered susceptible although the disease pathology may be different depending on the level of susceptibility of the relevant host (Clubb, 1986; Alexander, 1995b; Alexander, 1996). Non-avian species such as insects, rodents, scavenging animals, reptiles and humans can also be infected with NDV, thereby acting as reservoirs for the virus (Alexander, 1995b).

ND has been reported as the cause of mortality in ostriches in zoos in Morocco and Frankfurt, an Italian circus and ostriches on an ostrich farm in Israel that were reared in close proximity to commercial poultry (Corrado, 1966; Palmer and Trainer, 1971; Samberg *et al.*, 1989; Huchzermeyer, 1994a). ND was first reported in ostriches in South Africa in 1993 near Addo in the Eastern Cape Province, an ostrich farm near Skeerpoort, Mpumalanga and Zeekoegat near Pretoria in Gauteng (Huchzermeyer, 1993). From 1994 to 1997 outbreaks in ostriches were reported in the regions of De Aar in the Northern Cape Province, Cradock in the Eastern Cape Province, Bloemfontein in the Free State Province and Somerset East, Oudtshoorn, Mossel Bay and George in the Western Cape Province. Except for the infection in

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Bloemfontein, all the other birds that were infected died (Coetsee, 1994; Morley, personal communication). Both young and older ostriches are susceptible to ND and those that do become infected usually die (Cross, 1991; Coetsee, 1994; Huchzermeyer, 1994a; Allwright, 1996).

2.2.2. TRANSMISSION AND INFECTION

Natural transmission of NDV depends on the availability of virus in an infectious form and although the level of susceptibility differs between host species, any susceptible species can act as a source of virus for other more susceptible species (Cross, 1991; Awan *et al.*, 1994; Alexander, 1997).

Transmission of NDV can occur either through vertical or contact transmission (Alexander, 1997). Vertical transmission occurs when NDV is transmitted from the hen to her progeny via the egg. Cracked or broken eggs can act as source of virus for newly hatched chicks and the virus may also penetrate the shell after laying. Infected embryos normally die long before hatching, but those with avirulent strains may still hatch (Awan *et al.*, 1994).

For contact transmission to be possible, birds have to be in direct contact with one another as virus may fail to spread to susceptible birds housed in either a distant or neighbouring pen. Close contact of birds on the other hand, allows the transmission of NDV at an alarmingly fast pace (Travers, 1994).

Natural transmission usually occurs either through inhalation or ingestion (Awan *et al.*, 1994) and the mode of transmission of NDV is dependent on the organs in which the specific virus strain multiplies. Infection and replication of NDV in the respiratory tract causes NDV to be shed in mucus, which in turn will be transmitted via aerosols and inhaled by susceptible birds (Travers, 1994; Alexander, 1997). Virus strains that replicate in the intestines on the other hand will be transferred by ingestion of contaminated faeces either directly or via contaminated food and water or even by inhalation of dried dust or faeces particles (Travers, 1994; Alexander, 1997).

Survival of virus in a dead host or its excretions allows successful spread of the virus. NDV can survive on infected carcasses for several weeks at cold ambient temperatures and for several years if kept frozen. NDV can also survive in water and at 37°C NDV can retain its infectivity in faeces for over a month and even longer at cooler temperatures. However, the harsh summer climates found in South Africa

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usually allows NDV to retain its infectivity for only 1 – 2 weeks (Awan *et al.*, 1994; Travers, 1994; Alexander, 1996).

Important factors that have been implicated in the spread of NDV during epizootics include (Travers, 1994; Alexander, 1996; Alexander, 1997):

- movement of live birds and other animals;
- movement of people and equipment;
- movement of poultry products such as dead birds, faeces for fertilizer and feathers (Considine, 1993);
- airborne spread may play a significant role in ND epidemics given suitable climatic conditions and close proximity of poultry farms (Alexander, 1995b);
- contaminated feed and water;
- vaccines and
- wild birds or animals that can either carry the infection or be responsible for mechanical transfer. This might, however, be more of a factor with birds kept on open range such as ostriches. Wild birds that can act as carriers or reservoirs of the virus are waterfowl, parrots, songbirds, owls and birds of prey (Verwoerd, 2000).

After natural exposure the incubation period for NDV varies from 2 – 15 days (average 3 – 6 days) (Travers, 1994). The incubation period varies depending on the infecting strain, the susceptibility of the host, host age and immune status, concurrent infections, route of exposure and virus dose (Alexander, 1997).

In South Africa expansion in both commercial poultry and ostrich industries have brought these two industries closer. With the first recorded outbreaks in ostriches in 1993, confirmed ND mortalities could be traced back either directly or indirectly to commercial poultry, or intensive ostrich farming districts where there was no control over movement of birds (Verwoerd, 1995). The 1993 outbreak of virulent ND in South Africa occurred in poultry and from there, spread to ostriches. As the epidemic spread to poultry in neighbouring countries, outbreaks also occurred in ostriches in Namibia, Botswana and Zimbabwe, indicating that either commercial or village poultry flocks that are in close vicinity to ostrich farms can act as reservoirs for NDV

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(Huchzermeyer, 1997; Verwoerd, 2000). Ostriches in a flock, that are infected with ND, are not seen as a significant source of infection for other ostriches and the transmission of NDV amongst ostriches therefore seems to be influenced by the proximity of susceptible or infected poultry as well as movement of ostriches from farm to farm or between areas (Huchzermeyer, 1994a). The dissemination of ND by humans or wild birds, normally found around the ostrich feedlots, cannot be excluded (Huchzermeyer, 1997; Verwoerd, 2000). Thus, control over the movement of humans and other animal or birds is an important factor in preventing ND infection and spread (Allwright, 1996).

Transmission of NDV between ostriches is mainly through drinking water or feed and is caused by saliva from infected birds that drip into the water- or feed-troughs. NDV is then ingested by susceptible birds drinking and eating from the same troughs. This, however, results in a slower transmission of ND than with respiratory transmission resulting in infections being restricted to a single group or camp. Virus may also be found in the faeces and urine of infected birds (Huchzermeyer, 1993; Coetsee, 1994).

2.3. PATHOLOGY

Clinical signs differ according to the relevant infecting pathotype and disease can vary from being extremely pathogenic to inapparent (Alexander, 1996). Disease signs are also affected by factors such as bird species, host immune status and age, environmental conditions and the presence of concurrent diseases or organisms such as mycoplasma species (Beard and Brugh, 1975). Of all the possible factors that can influence disease signs, no specific sign can be regarded as pathognomonic for ND (Alexander, 1996; King, 1996). In chickens, especially chicks, virulent disease can appear suddenly with high mortality and no clinical signs. In such cases the first indication of disease is sudden death (Beard, 1992; Alexander, 1996). Typical clinical signs seen in poultry depends on the route of virus contact and in turn the area that is infected by the specific NDV strain (Beard and Brugh, 1975; Travers, 1994; King, 1996).

In VVND signs include listlessness, increased or difficult respiration, depression, weakness and eventually death (Travers, 1994; Alexander, 1996). VVND infections

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may cause oedema around the eyes and head, green diarrhoea with nervous signs such as muscular tremors, torticollis, paralysis of legs and wings and opisthotonos may be apparent prior to death and in fully susceptible birds, mortality reaches 100% (Alexander, 1996; Alexander, 1997). Birds that survive initial VVND infections develop severe neurological signs after two weeks making the disease indistinguishable from NVND (Beard, 1992).

NVND infections are marked by severe respiratory disease followed by neurological signs and a drop in egg production with eventual cessation of egg laying. Although morbidity may reach 100%, mortality is generally lower (Alexander, 1996; Alexander, 1997).

Infections with mesogenic strains usually cause severe respiratory infections and there may be a drop in egg production. Nervous signs can be present, although not common. Mortality is usually low (50% or more), but may be affected by exacerbating conditions such as concurrent infections (Alexander, 1996; Alexander, 1997).

Lentogenic strain infections usually cause no disease in adults, but in young birds serious respiratory disease can be seen with resulting mortality. Concurrent diseases may, however, result in signs similar to the more virulent strains (Alexander, 1996; Alexander, 1997). Some lentogenic strains infect the intestinal tract, but once again there are essentially no clinical signs (Beard, 1992).

Gross lesions are once again dependent on the pathotype of the infecting virus and the organs that are affected usually relate to the disease signs seen. There are, however, no lesions that are pathognomonic for ND and lesions may be totally absent (Alexander, 1996; Alexander, 1997). VVND can be distinguished from NVND by haemorrhagic lesions in the intestine (particularly the proventriculus) of infected chickens (Travers, 1994; Alexander, 1996). Generally lesions are not seen in the central nervous system, regardless of the pathotype, but lesions that have been reported include neuronal degeneration (Alexander, 1996). Gross pathological changes are seldom present in the respiratory tract, but if observed they consist of haemorrhagic lesions together with marked congestion of the trachea and airsacculitis may be present with thickening of air sacs and catarrhal or caseous exudates (Travers, 1994; Alexander, 1996; Alexander, 1997).

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The route of infection in ostriches has an influence on the resulting clinical signs seen. If ostriches are kept indoors or in closed chick-rearing units, infections tend to be via the respiratory route with subsequent respiratory disease that is rapidly spread between ostriches with high mortalities. If kept outdoors, they usually contract the virus via the oral route from faeces or water and clinical signs seen in this case are nervous symptoms with infections spreading slowly (Huchzermeyer, 1997). In general, typical respiratory symptoms, as seen in poultry, rarely occur in ostriches and therefore respiratory spread is not common in ostriches (Coetsee, 1994).

Typical ND symptoms in ostriches include tremors of the neck, head and legs and later on the whole body. The neck is limp causing the head to be held to one side with shaking from time to time, and later the neck hangs low between the legs and the bird is unable to lift its head with resulting oedema of the head and neck. Birds lose their coordination, swaying from side-to-side and eventually become paralyzed. Some birds may drool at the mouth and have watery eyes and a runny nose (Huchzermeyer, 1993; Coetsee, 1994; Huchzermeyer 1994a; Allwright, 1996).

Symptoms can develop within 4 – 10 days after infection depending on the infecting strain and infected ostriches can die within 24 – 36 hours after the first symptoms are seen (Coetsee, 1994; Allwright, 1996). Deaths vary from single birds to more than 30% in flocks whilst mortality in poultry can easily reach 100% (Coetsee, 1994; Huchzermeyer, 1994a). The low level of mortality in ostriches is probably due to the lack of respiratory involvement in the transmission of the virus (Coetsee, 1994; Huchzermeyer, 1994a). However, if no control measures are taken against infections with velogenic ND, mortalities in ostriches can reach 100% (Verwoerd, 1995).

Post-mortems done on ostriches that died in an Italian circus with typical symptoms of ND had gross lesions such as haemorrhagic tracheitis, oedema and congestion of the lungs, petechial haemorrhages on the epicard, haemorrhages in the mucosa of the proventriculus and haemorrhagic enteritis (Huchzermeyer, 1994a). In ostriches diagnosed with ND in South Africa gross lesions were minimal and non-specific, but can include lymphocytic pancreatitis, focal cardiomyopathy and oedema of the brain (Huchzermeyer, 1993; Huchzermeyer, 1994a).

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2.4. IMMUNITY

Both innate and specific immune processes influence host resistance to NDV infections. Non-specific immunity comprises many cellular and humoral processes. The ability of a bird to react to NDV infections is mainly influenced by genetic differences (some species are more susceptible than others) and host age. Usually a bird displays an increased resistance with age and although the effect of age can be related to non-specific factors, it may also be related to maturity of the immune system that mediates the specific immune response (Kramer, 1972; Beard and Brugh, 1975).

Specific immunity is an acquired response that can be manipulated through vaccination. This immunity is comprised of B-cells that mediate humoral antibody responses and T-cells that mediate a cellular response (Beard and Brugh, 1975; Vainio and Imhof, 1995; Reynaud and Weill, 1996).

The main types of immunity involved in NDV infections are the following:

i) Cell-mediated immunity:
After the nucleocapsid of NDV gains entry into the host cell, virus replication takes place within the cell cytoplasm. The initial immune response to the intracellular organism is cell-mediated and may be detected as early as 2 – 3 days after infection (Meulemans, 1988; Alexander, 1997).

ii) Humoral immunity:
If birds survive the initial NDV infection, antibodies can be seen in the serum within 6 – 10 days. The level of this immunity depends on the infecting strain and virus dose and generally peaks at 3 – 4 weeks after which it gradually declines (Alexander, 1997). Serum antibody levels are generally seen as a reliable and practical serological indicator of immunity (Meulemans, 1988).

iii) Local (Mucosal) immunity:
The primary site of infection for NDV is the ocular-nasal route and therefore local immune mechanisms play an important part of NDV epidemiology (Aitken and Parry, 1976). It is assumed that, in the case of vaccination, local application of the antigen is the best method for stimulating a mucosal antibody response (Waldman *et al.*, 1983).

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iv) Passive (Maternal) immunity:

Hens pass on NDV antibodies to their progeny via the egg yolk. Passive immunity does not necessarily prevent infection, but causes a variable modification of infection thereby decreasing its intensity (Bornstein *et al.*, 1952). Maternal immunity provides the developing chick with protective antibodies and should be taken into account when timing primary vaccination (Rehmani and Firdous, 1995). The reason for this is that maternal antibodies, together with the relatively low level of immune competence of the newborn chick, can interfere with vaccine antigens administered systemically (Kramer, 1972; Čajavec *et al.*, 1995; Alexander, 1997). Passive immunity in the chick can be manipulated by effective vaccination of the hens, thereby producing a better and more extended passive immunity in the progeny (Giambrone and Closser, 1990; Van Eck, 1990a).

2.5. DIAGNOSIS

For the diagnosis of ND one cannot rely on clinical signs and lesions alone, because the immune status and susceptibility of the host to a specific NDV strain may alter the clinical picture resulting in clinical signs that are similar to other poultry diseases (Cross, 1991). In addition to this, birds may even die without any obvious signs or lesions and therefore the diagnosis of infection is dependent on both virus isolation and identification (Beard, 1992; Travers, 1994; Alexander, 1996; Allwright, 1996; Alexander, 1997).

2.5.1. VIRUS ISOLATION

Regardless of clinical signs, samples from live birds should include both cloacal and tracheal swabs. In dead birds, samples should consist of intestines and intestinal contents, tracheas, organs and any tissues obviously affected or associated with clinical signs (e.g. brain samples if nervous signs are present). NDV contained within these samples can be grown in a variety of cell systems and subsequently isolated (Alexander, 1996; Alexander, 1997).

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2.5.2. VIRUS IDENTIFICATION

Conventional characterization of NDV isolates involve the assessment of pathogenicity of virulence and uses the following techniques (Alexander, 1996; King, 1996):

i) Mean death time (MDT) in eggs: inoculation of embryonated chicken eggs to determine a MDT.

ii) Intracerebral pathogenicity index (ICPI) in day-old chickens: inoculation of one-day old chickens to determine ICPI.

iii) Intravenous pathogenicity index (IVPI) in six-week old chickens: inoculation of six-week old chickens intravenously to determine IVPI or intracloacal inoculation of 6 – 8 week old chickens for differentiating between viscerotropic and neurotropic velogenic viruses.

Ultimately the isolation and identification of NDV is clear-cut proof of its presence in an infection, but the drawback of using these methods for pathotyping is that these tests firstly require specific pathogen free poultry. Secondly, it takes time to carry out and thirdly, does not always give a clear indication of the pathogenicity of a specific isolate, specifically those with intermediate virulence. The advent of molecular biology techniques has simplified this process allowing a more rapid identification and confirmation of NDV infections. Other tests that have been developed include (Della-Porta and Spencer, 1989; Considine, 1993; Verwoerd, 1995; Verwoerd, 2000):

- the use of monoclonal antibodies that can differentiate between isolates on the basis of antigenic differences;
- determination of differences between protein sequences and
- investigation of differences in gene sequences using the polymerase chain reaction (PCR).

2.5.2.1. Serology

Serology is mostly used for the detection of antibody responses in serum samples and the most common techniques used include virus neutralization (VN), haemagglutination inhibition (HI) and enzyme-linked immunosorbent assay (ELISA) (Alexander, 1996). The presence of antibody to NDV gives little information on the

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infecting strain and in this respect serology is of little diagnostic value although the ability to demonstrate infection is often sufficient. Serology can, however, be used to evaluate either immune responses to specific vaccines or the effectiveness of a vaccination program (Alexander, 1997).

The HI test is the acknowledged reference method for determining antibody levels in domestic fowl towards NDV (Miers *et al.*, 1983; Czifra *et al.*, 1996). The advantage of this technique is its simplicity and therefore highly trained personnel or expensive equipment is not needed (Miers *et al.*, 1983). Disadvantages of this method include, low sensitivity regarding the equal detection of antibody to all strains of NDV, non-specific agglutination of erythrocytes and cross-reaction between different serotypes resulting in false positive and false negative results, low reproducibility between different laboratories, time consumption and the requirement of serial serum dilutions (Miers *et al.*, 1983; Wilson *et al.*, 1984; Verwoerd, 2000).

ELISA has replaced the use of HI in many laboratories and was found to be successful in screening of antibody activity to NDV (Snyder *et al.*, 1982). ELISA is generally more sensitive than the HI test and is able to detect antibody in field samples not detectable by HI (Snyder *et al.*, 1982; Miers *et al.*, 1983; Williams *et al.*, 1997; Verwoerd, 2000). Della-Porta and Spencer (1989), on the other hand, have found the ELISA to be just as sensitive and specific as the HI test. The advantage of an ELISA is that the assay can be standardized in the case of commercially available kits, which enhances its effectiveness. Other advantages include a single serum dilution, high specificity and sensitivity and adaptability for studying various disease agents and immunological responses. It also requires very little sample material and tests can be semi-automated if required, allowing a large number of samples to be tested with a single assay thus making the test more economical (Miers *et al.*, 1983; Snyder *et al.*, 1983; Wilson *et al.*, 1984; Erhard *et al.*, 1992; Czifra *et al.*, 1996).

2.6. PREVENTION AND CONTROL

In February 1984, ND returned to Britain's poultry houses after a six-year absence. The reintroduction of NDV was traced back to animal feed at the Liverpool and Birkenhead docks that was contaminated by feral pigeons infected with NDV (Anon, 1985). In South Africa, the 1993 outbreak of virulent ND in poultry occurred after a

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20-year absence of this disease (Verwoerd, 1995). Situations such as this emphasize the importance of having the necessary control policies in place.

ND is a notifiable disease in South Africa and any suspicion or actual incidence of this disease is required by law to be reported to the local state veterinarian. The state veterinarian will then immediately perform the necessary tests to confirm NDV presence as well as to identify the relevant strain (Anon, 1993; Alexander, 1994). In areas that are free of ND, the primary aim should be to prevent the introduction of the virus and effective control of NDV relies on good management and biosecurity practices as well as prophylactic measures such as vaccination (Meulemans, 1988; Travers, 1994; Alexander, 1995).

Directly after the 1993 outbreak of NDV in South Africa, strict control measures were implemented by the Department of Agriculture in an attempt to prevent further spread. Control measures included clinical monitoring and stock registers pertaining to mortalities, movements, vaccinations and general health status of flocks (Anon, 1993). Ostrich farmers were urged to start with a vaccination program as soon as possible (Anon, 1993).

The proximity of an ostrich farm to another or to a poultry farm will dictate the control management and biosecurity measures implemented against ND (Verwoerd, 1995). Effective control on an ostrich farm includes vaccination of surrounding poultry (within a specific radius), disallowing the keeping of any non-ratite and other susceptible birds on the ostrich farm and preferably keeping to a strict vaccination schedule for the ostriches (Huchzermeyer, 1997).

In the event of an outbreak, infected ostriches should immediately be separated from other birds. The pen where infected birds were held should be sterilized along with their feeding- and water-troughs (Coetsee, 1994). Birds with no visible symptoms should be vaccinated first followed by those of which the disease status is uncertain (Coetsee, 1994).

If NDV is diagnosed on an ostrich (or poultry) farm, according to regulations the farm itself is put under quarantine for 90 days. Surrounding farms, within a 10 km radius, are quarantined for 30 days and all birds in this area should be vaccinated against NDV immediately. No birds may be moved out of this area for the 30 day quarantine period. Also, no birds within this 10 km radius may be slaughtered for export

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purposes and even in the absence of an outbreak all slaughter ostriches should be vaccinated 30 days before slaughter (Coetsee, 1994). This period of 30 days after vaccination is deemed sufficient to eliminate the danger of NDV being exported in the meat obtained from these birds (Huchzermeyer, 1997). In addition to this, birds should be inspected for any nervous symptoms or any other typical ND signs to prevent possible infected birds from being slaughtered and their meat, which might contain NDV, exported (Huchzermeyer, 1997). During an outbreak slaughter birds destined for export should be regularly checked by means of blood tests and post-mortems to ascertain their disease status (Anon, 1993).

Monitoring and surveillance of ND in ostriches is important not only because of increased ostrich production and therefore more intensive farming conditions, but due to ND posing a threat to animal industries in countries importing ostrich products (Huchzermeyer, 1997). In addition to this is the potential effect that an outbreak of ND in ostriches can have on poultry trade at an international level (Ley *et al.*, 2000).

2.6.1. CONTROL BY VACCINATION

When deciding to vaccinate, consideration should be given to the reason for vaccinating, as this will influence the eventual vaccine regimen chosen. Emergency vaccination in the face of an outbreak has the purpose mainly to reduce mortalities and losses rather than providing total protection against disease. This is especially the case in instances where either the active or passive immune status of chicks or flocks is uncertain. Some birds may already be harbouring the virus without clinical disease or mortality and vaccination under these unfavourable conditions tends to be less effective (Lim *et al.*, 1993; Alexander, 1994). Roy and Balasubramaniam (1992) stressed the importance of correct vaccination as faulty vaccination can lead to infections or outbreaks. Therefore, vaccination schedules should be followed strictly and with the utmost care and antibody titres estimated regularly in order to control the disease. It should be remembered, however, that despite vaccination, virulent NDV strains may still replicate in the host bird, but the clinical signs will be greatly diminished (Alexander, 1994).

Aspects that should be considered in the choice of vaccine and in devising vaccination programmes include (Travers, 1994; Alexander, 1995b; Alexander, 1996):

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- Age of the birds. The first vaccination that birds receive provides protection to chicks with either low-grade maternal antibody levels or with decreasing maternal antibody levels (Shivdekar, 1995). In chicks, maternal antibodies persist in sera until about 3 weeks of age (Čajavec *et al.*, 1995) and they rarely carry their parental immunity beyond one month (Beaudette and Bivins, 1953).
- The current disease situation.
- Disease control policies.
- Availability of vaccine and its immunogenicity.
- Live or inactivated vaccine as this, depending on the route of administration, could influence the type, tempo and duration of an immune response (Gough and Alexander, 1973; Eidson *et al.*, 1976; Rehmani and Firdous, 1995).
- Route of vaccination. An important requirement of any vaccine is that it should induce an immune response in the right place. If resistance to mucosal infections is needed, stimulation of secretory immunoglobulin A (IgA) production will be more appropriate, whilst serum IgG or IgM will be of more value in systemic infections (Mims, 1987).
- Virulence of the vaccine strain (in the case of live vaccines) not only for the recipient host, but also because of the possibility of lateral spread of vaccine virus. When birds are vaccinated with a live vaccine they become infected and in the process they can excrete virus despite remaining apparently healthy. This aspect should be taken into account during control measures against ND as vaccination can contribute to the development of immune carriers (Clubb, 1986; Alexander, 1995b).
- Presence of concurrent infections.
- Influence of other vaccines administered either before, after or simultaneously.
- Presence of maternal immunity. Local vaccination with either live or inactivated vaccines is not influenced by maternal antibody (Zakay-Rones and Levy, 1973). Systemic vaccination on the other hand is negatively influenced by the presence of maternal antibodies.

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- Flock size. Individual methods of vaccine application are not economically feasible in large flocks (Meulemans, 1988).
- Lifespan of birds. This will influence the number of times a bird needs to be vaccinated in order to achieve maximum immunity, as well as the time intervals between successive vaccinations.
- Available labour. If required, this will allow the individual vaccination of large flocks.
- Climatic conditions. This will influence the stability of a vaccine and therefore the storage conditions required. Inactivated vaccines tend to be more stable than live vaccines (Mims, 1987).
- Past performances of vaccination programmes.

In preventing the introduction of NDV, vaccination should under no circumstances be seen as an alternative to good management, biosecurity or hygiene practices (Alexander, 1994).

2.6.1.1. Live vs inactivated vaccines

Live vaccines:

The efficacy of a live virus vaccine depends on its invasiveness or power to multiply sufficiently within the bird in order to stimulate an immune response (Meulemans, 1988). Initially at the time of administration the antigenic mass is small, but increases many thousands of times following multiplication of the microorganism in the body. The effective dose is thus greatly amplified and therefore only a single dose is needed to produce satisfactory immunity (Mims, 1987). Live vaccines thus allow a single application, which reduces costs and untoward vaccine reactions and avoids stress of repeated application during rearing (Box *et al.*, 1978; Čajavec *et al.*, 1995). In turkeys, a live lentogenic NDV vaccine was shown to multiply to high titres in the trachea following aerosol exposure, but viral replication was associated with marked damage to the tracheal mucosa. In order to avoid this, inactivated vaccines would be more effective (Kelleher *et al.*, 1988).

In the production of live vaccines for chickens, there is a preference for lentogenic strains such as Hitchner B1 and La Sota due to their effectiveness and safety

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compared to mesogenic strains such as Roakin and Van Roekel (Meulemans, 1988). A rapid onset of immunity would be invaluable in controlling the spread of ND during an epizootic and this can be achieved by using live lentogenic vaccines such as B1 or La Sota that are able to induce resistance to challenge within a few days of vaccination before circulating antibody has developed (Gough and Alexander, 1973; Bell *et al.*, 1991). La Sota generally confers better protection than B1 due to La Sota having a greater spreading potential from chick to chick than B1 and birds that have been missed in initial application have the potential of being exposed during the period of spread (Goldhaft, 1979). Due to post vaccination respiratory symptoms caused by La Sota, it is better not given before 21 days (chicks are then better able to sustain the stress of vaccination). B1 vaccines can be given earlier as it causes little or no respiratory reactions (Shivdekar, 1995).

Considering aspects such as route of administration and dosage, B1 and La Sota vaccines are not efficient in all cases despite the fact that they are the most widely used commercial vaccines. Other live vaccines that may be used with success include V4 and the F-strain vaccine (Meulemans, 1988; Shivdekar, 1995).

Live vaccines can be administered either individually (intranasal, ocular or beak dipping) or through mass vaccination via drinking water and aerosol or spray administration (Meulemans, 1988). Individual application does seem to result in more consistent protection although this form of application is less economical (Meulemans, 1988). If locally applied, live attenuated vaccines stimulate a more solid immunity than do inactivated viral vaccines (Waldman *et al.*, 1983). Lentogenic strains have an affinity for the respiratory epithelium and are thus more effective when applied via the respiratory route (ocular or nasal instillation). Ocular vaccination results in a higher antibody response than via drinking water and results in a longer duration of immunity and therefore a higher level of protection (Shivdekar, 1995). Aerosol application of live La Sota vaccines induces both systemic and local immunity, although aerosol application can cause undesirable post vaccination reactions such as rhinotracheitis or conjunctivitis (Van Eck, 1990b).

Inactivated vaccines:

Inactivated vaccines must be given in repeated doses, because during the primary immune response, antigen is naturally being disposed of. There is thus a need for additional boosts to stimulate a secondary immune response with subsequent

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memory (Mims, 1987). Immunity with inactivated vaccines is consequently established slowly (Van Eck, 1987).

Oil-based inactivated vaccines have been found to be more immunogenic than aluminium hydroxide inactivated vaccines. Oil-based vaccines, however, elicit severe tissue reactions in ostriches and intramuscular vaccination may lead to formation of abscesses or tissue necrosis with resulting downgrading of carcasses at the abattoir (Verwoerd, 1995; Burger, personal communication).

Inactivated vaccines are usually individually applied either subcutaneously or intramuscularly (Meulemans, 1988). Individual vaccination of inactivated vaccines is expensive and labour intensive. Inactivated vaccines are therefore rather used as booster vaccines in birds that have previously received a live vaccine. This results in higher and more persistent antibody levels than in birds only receiving live vaccine (Van Eck, 1987; Meulemans, 1988).

Systemically applied inactivated vaccines provide high systemic immunity, but this usually does not induce local immunity in the respiratory or digestive tract (Van Eck, 1990b). Zakay-Rones *et al.* (1971) showed inactivated NDV vaccine, administered intranasaly, to elicit a local antibody response in the lungs. However, according to Yoshida *et al.* (1971), intratracheal and intranasal administration of inactivated NDV vaccines elicited less antibody response in mucosal secretions than with live vaccine.

2.6.1.2. Vaccination efficacy

A summary of the efficacy of different vaccines and vaccination schedules as discussed hereafter is shown in Table 2.1. Antibody titres are expressed as HI or VN values which are not always directly comparable.

Parry and Aitken (1973) measured local antibody responses (IgA) in the respiratory tract of chickens following exposure to live and inactivated oil emulsion (IOE) B1 NDV vaccine. The live vaccine induced maximal tracheal VN titres of ~140 four days after vaccination which subsided rapidly, whilst serum VN titres reached ~320, 18 days after vaccination. If the dilution factor of 1:10 of the tracheal samples is taken into account, the mucosal VN titres reached, after live tracheal vaccination, where much higher than the serum VN titres. The inactivated vaccine elicited a much higher serum antibody response (VN ~1280) than the live vaccine, whilst tracheal antibody titres never exceeded 20% of corresponding serum levels. Parry and Aitken (1973)

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ascribed the tracheal immunity to locally produced IgA. After challenge with a live La Sota vaccine (2 x $10^{7.5}EID_{50}$/eye drop), local immunity could limit, but not prevent infection. It was, however, suggested that a local immune response might be better adapted to dealing with repeated low levels of infection, than with a single large challenge dose (Parry and Aitken, 1973). Zakay-Rones *et al.* (1971) used an Isreali strain of live NDV for vaccination of chickens via the intranasal or intramuscular routes and obtained comparable results to those of Parry and Aitken (1973) in that intranasal vaccination leads to similar mucosal and serum antibody levels. Intramuscular vaccination resulted in low mucosal antibody levels and high serum antibody levels. They did, however, not assess the efficacy of this vaccination by challenging the vaccinated birds with live velogenic NDV.

Aerosol application of a live B1 vaccine can induce both systemic and local immunity, although protection against challenge with a virulent NDV strain is present before serum HI can be detected (Gough and Alexander, 1973). Primary stimulation, using a live La Sota vaccine in combination with an injection of an IOE (La Sota) vaccine, has been shown to provide protection in chickens and turkeys and thereby avoiding additional revaccination during rearing (Box *et al.*, 1978; Cajavec *et al.*, 1995).

For an inactivated vaccine (La Sota) to be effective if applied locally, vaccine dosage is important as indicated by Van Eck (1990b), where an increased dosage resulted in increased survival after challenge. Kelleher *et al.* (1988) showed IOE (La Sota) vaccinated birds to be susceptible to NDV respiratory infection as measured by virus isolation. Little secretory antibody formed in the respiratory mucosa of turkeys inoculated with an inactivated NDV vaccine by a parenteral route. Although the V4 strain of a live NDV vaccine has been reported not be as immunogenic as B1 and La Sota strains, this vaccine has been used with success with 97% protection achieved after challenge (Bell *et al.*, 1991).

2.6.1.3. Vaccination regime

In NDV endemic areas such as South Africa, it is recommended that ostrich chicks be vaccinated via eye drop or spray administration of a live vaccine (La Sota) followed by a subcutaneous injection of an inactivated vaccine 3 weeks later (Huchzermeyer, 1994a). This should be repeated every 6 months during rearing and

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Table 2.1 Vaccine efficacy using different vaccines and immunization routes

AUTHOR	SPECIES	MATERNAL ANTIBODY	AGE AT ADMINI-STRATION	VACCINE ROUTE	VACCINE DOSE	MAXIMUM IMMUNE RESPONSE		CHALLENGE	PROTECTION
						SERUM	MUCOSAL		
Box *et al.* (1978)	Turkeys	Positive HI: 4.9 at day-old	1 day 10 weeks	Intraocular & Intranasal	Live La Sota & IOE 0.5ml IOE 0.5 ml	HI: 8.6 HI: 11.8	NA	NA	NA
Van Eck, (1990b)	Chickens	Positive	1 day	Intracheal & oculonasal	Inactivated La Sota 6750 µg 675 µg 338 µg	 HI: 4.7 HI: 4.0 HI: 4.0	NA	Oculonasal 28 days 21 days 21 days	MA delayed IR 12/12 = 100% 8/10 = 80% 5/10 = 50%
Cajavec *et al.* (1995)	Chickens	Positive	1 day	Oculonasal	Live La Sota $1x10^{6.2}EID_{50}$ Live & Inactivated La Sota 0.2 ml	HI: 22 HI: 22	NA	42 Day 72 Day 42 Day 74 Day	9/10 = 90% 8/10 = 80% 10/10 = 100% 10/10 = 100%
Bell *et al.* (1991)	Chickens	Negative	11 day	Aerosol	Live V4 $1x10^{6}EID_{50}$	HI: 4.19	NA	14 days 21 days 31 days	77% 97% 92%

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Table 2.1 Continues

AUTHOR	SPECIES	MATERNAL IMMUNITY	AGE AT ADMINI-STRATION	VACCINE ROUTE	VACCINE DOSE	MAXIMUM IMMUNE RESPONSE		CHALLENGE	PROTECTION
						SERUM	MUCOSAL		
Kelleher *et al.* (1988)	Turkeys	Negative	20 days	Sub-cutaneous Oculonasal	IOE 0.5 ml Live LaSota	HI: 32 HI: 16	NA	Oculonasal & Aerosol	100% 100%
Parry and Aitken (1973)	Chickens	Negative	3 weeks	Oculonasal Sub-cutaneous	Live B1 $2x10^{6.4}EID^{50}$ IOE B1 0.5 ml	VN: 320 (Day 18) VN: 300 (Day 18)	VN: 140 (Day 7) VN: 19 (Day 15)	Eye-drop	Infection limited but not prevented
Gough and Alexander (1973)	Chicken	Negative	4 weeks	Drinking H_2O Intraocular Aerosol Aerosol	B1 B1 Inactive	HI: 5.0 (Day 17) HI: 4.3 (Day 17) HI: 2.3 (Day 17) HI: <2.0 (Day 17)	NA	Aerosol	100% 100% 100% 0%
Zakay-Rones *et al.* (1971)	Chickens	Negative	6-7 weeks	Intramus-cular & Intranasal	NDV Israel strain	HI: 640 HI: 5	HI: 5 HI: 5	NA	NA

NA: Not Assessed **MA:** Maternal Antibody **IR:** Immune Response

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once a year in breeding birds. Verwoerd (1995) recommended a vaccination program consisting of a La Sota (eye drop) vaccine given at 4 – 6 weeks, followed three weeks later with an inactivated oil adjuvant. If live vaccines are given to birds younger than 4 – 6 weeks, they may exhibit side effects such as fever, conjunctivitis and sinusitis. In ostriches older than 6 months it is more practical to give both vaccines simultaneously since ostriches at this age are difficult to handle (Verwoerd, 1995). Madeiros (1997) recommended vaccination schedules for ostrich chicks with and without maternal antibody. For ostrich chicks without maternal antibody, one eye drop of live vaccine, together with 0.5 ml of inactivated vaccine subcutaneously administered to two week-old chicks was recommended. Chicks with adequate maternal antibody levels need only be vaccinated at 45 days of age with 3 ml of inactivated vaccine (Madeiros, 1997). Despite the fact that Madeiros (1997) reported the protection of ostrich chicks by maternal antibodies, the determination of ostrich maternal antibody transfer to the egg yolk was not documented. In the above vaccination schedules for ostrich chicks with live NDV vaccines, it is assumed that adequate levels of mucosal immunity are produced. The efficacy of these vaccination schedules in producing mucosal immunity, i.e. IgA antibodies, however, has not been documented.

In a previous study, we examined the efficacy of vaccines and vaccination schedules against ND for both slaughter ostriches and ostrich chicks as prescribed by the Klein Karoo Co-operative to the ostrich farmers in the Oudtshoorn area (Blignaut *et al.*, 2001). In the first of two vaccination trials, slaughter ostriches were each vaccinated subcutaneously with 0.5, 1 and 1.5 ml of an inactivated alum-precipitated La Sota vaccine at 2.5, 3.5, 7 and 13 months of age. From the results it was deduced that an immunization schedule for slaughter ostriches, which would provide adequate protection against ND, should include using Lomavac (inactivated alum-precipitated La Sota vaccine) at 0 – 2 months (1 ml), at 2 – 6 months (2 ml), after reaching 6 months of age (3 ml) and at least 30 days (3 ml, and no longer than 6 months) before slaughter.

In the second trial, the efficacy of vaccination of ostrich chicks, using live and inactivated La Sota vaccines, was assessed. Chicks were vaccinated at 5 weeks and again at 12 weeks of age with either live vaccine (one drop in each eye), or inactivated vaccine (1 ml) or a combination of live and inactivated vaccines. Ostrich

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chicks that received the inactivated vaccine were able to produce a humoral immune response, but only after the second vaccination. The chicks that received only live La Sota vaccine failed to produce a significant humoral immune response. However, the mucosal immune response was not assessed in this study.

It was thus decided to assess the transfer of maternal immunity from ostrich hens to the egg yolk as well as the mucosal immunity produced in ostrich chicks in response to NDV vaccination. These aspects will be addressed in the next chapter.

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CHAPTER 3:

Characterization of maternal and mucosal immunity to NDV in ostrich chicks

3.1. INTRODUCTION

Immunity in ostrich chicks can be influenced by both active and passive immunization. Passive immunity in chickens is transferred from the hen to the chick via the egg yolk. By hyperimmunization of a hen, the levels of maternal antibody, which may be transferred to the yolk and eventually to the progeny, can be increased thereby providing the chick with protective levels of maternal antibodies (Giambrone and Closser, 1990; Sharma, 1999). Although maternal antibodies will not prevent infection of the respiratory system, it will reduce the pathogenicity of an existing infection in chicks (Mockett *et al.*, 1987). In chickens, anti-NDV antibodies are transferred from the hen to the chicks via the egg yolk (Bornstein *et al.*, 1952; Heller *et al.*, 1977). Although it is accepted that maternal antibodies are transferred from the ostrich hen to the chick (Madeiros, 1997), this has not been shown experimentally.

Active immunization can play an important role in cases where either the passive or active immune status of chicks is uncertain (Alexander, 1994). In chickens, the primary site of infection for NDV is the respiratory or oral route and for this reason, emphasis has been placed on the local application of live NDV vaccines (Ewert *et al.*, 1979). Local application of vaccine strains, such as Hitchner B1 and Ulster 2C, have been reported to induce resistance to respiratory challenge within a few days of vaccination and that this immunity was present even before circulating antibodies developed (Bell *et al.*, 1991). Local antibody levels has also been shown to correlate better with protection to respiratory challenge than serum antibody levels (Holmes, 1979). The major antibody in locally produced immune responses is IgA. Local IgA antibodies on the mucosal surface of the respiratory tract play a critical role in inhibiting primary replication of NDV, resulting in protection from subsequent systemic infections (Takada and Kida, 1996). In the previous chapter, the use of live vaccines in various vaccination schedules for ostrich chicks was discussed. However, in none of these studies was the efficacy of these vaccination schedules in producing mucosal immunity assessed.

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The specific objectives of this study were therefore as follows:

1) The isolation and characterization of ostrich serum IgG and ostrich yolk IgY, with a view to establishing whether they were immunologically identical and therefore could both be detected using an ELISA to detect serum IgG anti-NDV antibodies.
2) An assessment of the transfer of maternal anti-NDV antibodies from the ostrich hen to the egg yolk using this ELISA.
3) The isolation and characterization of ostrich mucosal IgA and the production of secondary anti-ostrich IgA antibodies.
4) The development of an anti-NDV ELISA for the detection of mucosal IgA anti-NDV antibodies using the secondary anti-ostrich IgA antibodies.
5) An assessment of the mucosal immune response (IgA) of ostrich chicks to live La Sota vaccine administered locally using this ELISA.

The results obtained in achieving each of the objectives are described at the end of this chapter in the form of an independent manuscript. The manuscript is preceded by background information on certain aspects of the experimental design as well as strategies used in obtaining each of the above objectives.

3.2. PASSIVELY ACQUIRED IMMUNITY

Passive acquired immunity in poultry chicks is acquired from the hen via the egg yolk and this transfer process can be seen as the analogue of cross-placental transmission found in mammals (Rose *et al.*, 1974; Mockett *et al.*, 1987). The hen is equipped with a repertoire of antibodies that are a reflection of antigenic exposure during her life and transfer of these antibodies to the egg yolk subsequently provides the chick with a ready-made immunity until it can respond adequately to antigenic stimuli itself (Doll *et al.*, 1950, Heller *et al.*, 1977). Although the chick receives maternal antibody at a stage when its own lymphoid system is still developing, it does not mean that the chick is incapable of producing antibodies. However, during active immunization of the chick, maternal antibodies may interfere with this ability (Brambell, 1970).

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During growth of the egg (oocyte) in the follicle, vast stores of yolk are accumulated in the oocyte and are laid down in concentric layers. During this time, the follicle cells in the ovary selectively transfer antibodies (IgG) in large amounts from the maternal circulation into the developing oocyte, which is then incorporated into the yolk (Tressler and Roth, 1987; Erhard and Schade, 2001). After ovultation, the ovum passes down the oviduct during which the albumen (egg white), shell membranes and shell itself is formed before the egg is finally laid. The white of the egg has been reported to contain IgM and IgA (Rose *et al.*, 1974). These antibodies are present in the oviduct secretions and are acquired by the egg as it passes down the oviduct where the white is laid down. Although IgA and IgM are found mainly in the albumen, and IgG in the yolk, IgA and IgM have also been found in the yolk (Yamamoto *et al.*, 1975; Kaspers *et al.*, 1990). Yolk of freshly-laid eggs contain very little if any IgM or IgA and are most probably transferred from the egg white into the yolk sac contents during the last third of embryonic development (Kaspers *et al.*, 1991).

After the egg is laid, a network of blood vessels is formed around the yolk sac and yolk materials are then absorbed through the endoderm of the yolk sac and transported into the circulation of the developing embryo (Romanoff, 1960; Rose *et al.*, 1974; Tressler and Roth, 1987). Specific receptors on the yolk sac membrane have been found that are responsible for transporting the IgG from the yolk to the blood circulation of the chick (Mockett *et al.*, 1987). Transfer of IgA and IgM antibodies to the chick serum does not occur (Kaspers *et al.*, 1991). IgA and IgM have also been found in the developing embryo gut (Rose *et al.*, 1974). On the 11 – 12th day of incubation, the white from the albumen sac begins to flow into the amnion and mixes with the amniotic fluid. IgA and IgM present in the albumen are then probably ingested via swallowed amniotic fluid (Kaspers *et al.*, 1996).

Just before hatch the yolk sac, with remaining unabsorbed yolk, is retracted into the body of the embryo through the umbilicus and the yolk is then gradually absorbed during the next 5 – 7 days after hatch (Romanoff, 1960; Brambell, 1970). In chickens, maternal immunity is short lived, having a half-life of 4 – 5 days, and is therefore seldom detected in chicks beyond 5 – 6 weeks of age (Mockett *et al.*, 1987; Duchatel *et al.*, 1992).

In chickens, NDV-specific antibodies are passively transferred from immune hens to the egg yolk and this passive immunity in the chicks lasts from about 3 - 5 weeks and

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is known to interfere with active immunization (Bornstein *et al.*, 1952; Heller *et al.*, 1977). Whether maternal antibodies to NDV in ostrich hens are also transferred to the egg yolk has not been documented, although Madeiros (1997) has stated that properly vaccinated breeders will produce antibody protection in ostrich chicks for up to 45 days.

Chicken IgG is structurally different from that of mammalian IgG, as the heavy chain of chicken IgY has an additional constant domain instead of the hinge region of mammalian IgG (Warr *et al.*, 1995). For this reason, it has been proposed that chicken IgG should rather be called IgY. However, for the purpose of differentiating between ostrich serum and yolk IgG, yolk IgG will hereafter be referred to as IgY.

3.2.1. IgY ISOLATION

Quantitatively, IgY is the most common Ig in the egg and specifically the yolk, thus making egg yolk the ideal starting material for the isolation of IgY without IgM or IgA contamination (Kaspers *et al.*, 1991; Erhard and Schade, 2001). Akita and Nakai (1993) compared four different procedures for purification of Ig from hen eggs. These methods included isolation of IgY by means of polyethylene glycol and alcohol precipitation (PEG-Alc) (Polson *et al.*, 1985), dextran sulphate (Jensenius *et al.*, 1981), water dilution (WD) (Akita and Nakai, 1992) and xanthan gum (Hatta *et al.*, 1990). A comparison of these methods was done on the basis of yield, purity and activity of isolated IgY as well as ease of use and potential for scaling up. WD was found to be the best method on the basis of yield and purity of IgY. In their study, the highest activity of isolated IgY, as tested using ELISA, was achieved using the PEG-Alc method. As the IgY, to be isolated from ostrich egg yolk, would be measured using ELISA and because the PEG-Alc method allows the isolated IgY to better retain its activity, it was decided to use this method for ostrich yolk IgY isolation.

3.3. MUCOSAL IMMUNITY

The mucosal surfaces of the respiratory system, eyes, intestine, urinary tract and other tissues exposed to the external environment is largely covered by a layer of epithelial cells that form a barrier between the internal and external (antigen containing) environment (Jeurissen *et al.*, 1989). These mucosal surfaces firstly provide protection against antigens by non-specific mechanisms such as a

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continuous layer of epithelial cells, mucus and extreme pH values which prevent antigen from adhering to the mucosal surfaces (Neutra *et al.*, 1996). Secondly, it contains mucosal-associated lymphoid tissue that responds specifically to invading pathogens (Jeurissen *et al.*, 1989).

Virus neutralizing activity has been demonstrated in mucus and fluid washes of the respiratory tract of chickens following exposure to NDV and has been attributed to locally produced antibody (Parry and Aitken, 1973). Experimental evidence that has indicated the role of an active local immune system during NDV infections in fowl includes (Parry and Aitken, 1977):

i) a lack of correlation between circulating antibody titres and resistance to infection;

ii) chicks vaccinated locally (aerosol spray) with a live lentogenic NDV strain acquires greater resistance to challenge with a virulent strain than chicks vaccinated by parenteral routes;

iii) presence of anti-viral activity in external secretions (nasal and tracheal washes) following experimental infection with ND and

iv) the distribution of lymphoid cells in the respiratory tract and the paraocular and paranasal tissues, specifically the Harderian gland (HG) (Leslie *et al.*, 1971). Lymphoid tissue has also been described in the nasal turbinates, larynx and lower respiratory tract of normal and infected birds.

The idea of fowl having a mucosal immune system similar to that found in mammals was strengthened by the subsequent identification of IgA in the domestic fowl (Orlans and Rose, 1972; Parry and Aitken, 1977). IgA is the most abundantly expressed Ig in epithelial associated lymphoid tissues and is therefore the primary isotype found in the mucosal immune system where it is part of the first line of defence against external pathogens invading epithelial surfaces of the body (Underdown and Schiff, 1986; Mansikka, 1992). It protects mucosal surfaces by first preventing bacteria from adhering and thus penetrating the mucosal surface and second, neutralizing virus thereby preventing replication in mucous surface cells (Plaut, 1972; Allansmith *et al.*, 1983). Although IgA predominates in mucosal surfaces, other antibodies are also present (Plaut, 1972). Katz *et al.* (1974) found IgG, IgM and IgA in some airway

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washings and in other either IgA alone or IgA along with IgG. After local application of live NDV vaccine, IgM in tracheal washes is first detected at day 4 and peaks at day 7 after which levels decrease, whilst IgA concentrations in tracheal washes increases at day 4 and reached a plateau at day 7 (Al-Garib *et al.*, 2003). The presence of IgG in airway washings may be explained by an increase in capillary permeability in the trachea due to NDV infection. This causes passive transudation of serum proteins into the secretions of the respiratory tract and oral cavity. However, after local vaccination the majority of antibodies in secretions are of local origin, whilst serum antibodies account for a relatively small proportion of virus neutralizing activity in respiratory secretions (Aitken and Parry, 1976; Ewert *et al.*, 1977).

The primary site of infection for NDV is the oculo-nasal route and subsequent vaccination via these routes results in local antibody production on the mucosal surfaces of the respiratory tract. These antibodies play a critical role in inhibiting primary replication of NDV thus protecting birds from subsequent systemic infections with possible shedding of the virus (Takada and Kida, 1996). Secretory antibody levels are also known to correlate better with protection against viral infections of mucosal surfaces than do serum antibodies indicating the presence of a local immune system that functions independently from systemic immunity (Waldman *et al.*, 1983; Brandtzaeg, 1989).

3.3.1. IgA STRUCTURE

In avian species, IgA is present in various forms as monomeric, dimeric and even higher molecular aggregates, but dimeric forms mainly exist in external mucosal secretions whilst the monomeric form predominates in the circulation (Schranner and Lösch, 1986; Schranner *et al.*, 1987; Mansikka, 1992). Leslie and Martin (1973) found the polymeric form to predominate in both serum and secretions although the IgA:IgG ratios are higher in secretions than in serum.

In its monomeric form, IgA consists typically of two heavy and two light chains (Underdown and Schiff, 1986). In avian species such as the chicken the J-chain is assumed to be involved in formation of polymeric Igs and functions by joining monomeric subunits of IgA and IgM (Kobayashi *et al.*, 1973; Schranner and Lösch, 1986). The J-chain is linked to IgA by disulphide bonds and has a molecular mass of 23 000 daltons (Da) in human and rabbit (Halpern and Koshland, 1970).

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In secretions IgA exists mostly as a dimer combined with a carbohydrate rich secretory component (SC), which gives secretory IgA a different immunochemical and physiochemical property than serum or monomeric IgA (Katz and Kohn, 1976). SC is a glycoprotein that is synthesized by and expressed on cells of epithelial origin and acts as a receptor for locally produced dimeric IgA. The SC not only aids in the binding of IgA to the apical mucin layer, but also protects IgA from the degradative effects of proteolytic enzymes (Plaut, 1972). The complex formed by SC and IgA is internalized by the cell, transported across the cell in special vesicles and is discharged either intact or separated into the extracellular fluid. The type of bonding between IgA and SC and the stability of the complex differs between species (Karaca and Naqi, 1997). SC has, depending on the species, a molecular mass of about 80 000 Da (Rose *et al.*, 1981; Karaca and Naqi, 1997).

3.3.2. IgA ISOLATION

Substantial quantities of fowl Ig can only be prepared using complex recycling techniques or by starting with material rich in the relevant antibody class and deficient in others (Higgins, 1976). IgA was found to be amongst the chicken's repertoire of Ig present in serum, saliva, bile, intestinal and oviduct secretions and bronchial secretions (Bienenstock *et al.*, 1972; Lebacq-Verheyden *et al.*, 1974). Ion-exchange chromatography only, was unsuitable for the separation and purification of chicken Igs due to the fact that Igs precipitate in dilute buffers and polymerize in concentrated buffers (Kramer, 1972). Lebacq-Verheyden *et al.* (1974) quantitated the distribution of Igs in chicken serum and secretions and attempted to isolate IgA using ion exchange chromatography. They found, however, that during ion exchange chromatography all three Ig classes elute throughout most of the gradient and therefore has little application in the separation of fowl Ig. Lebacq-Verheyden *et al.* (1974) also concluded that the amount of IgA in serum did not allow for extensive use of serum for isolation purposes. Similarly, in humans, the serum IgA concentration is low compared to serum IgG and therefore gives low IgA yields contaminated with IgG (Roque-Barreira *et al.*, 1985).

In chickens, bile is the material of choice for isolation of chicken IgA as it is the predominant Ig in bile (Goel *et al.*, 1980). Another source is the HG, which is a paraocular gland that constitutes an important part of the secretory immune defence system in birds and contains densely packed plasma cells producing mainly IgA

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(Ewert, *et al.*, 1979; Powell *et al.*, 1979). The avian HG also plays a major role in producing lachrymal IgA in response to ocular vaccination with NDV (Russel and Koch, 1993). The ostrich HG could therefore be used for the isolation of IgA.

Another approach to the isolation of IgA is the use of affinity chromatography using immobilized jacalin. Jacalin is a D-Gal binding lectin, isolated from jackfruit seeds and is able to precipitate both human serum monomeric and polymeric forms of IgA (Roque-Barreira *et al.*, 1985). Cooper and Wright (2001) were able to separate human IgA and IgG, using affinity chromatography with jacalin, but found the IgA recovery to be low in the bound fractions with IgA remaining in the unbound fraction.

In the light of this background information, attempts were made to isolate ostrich IgA in this study. One of the major problems in isolating ostrich IgA was finding material rich in IgA with little contamination by other Igs. Ostriches do not have a gallbladder, and therefore do not produce bile. In preliminary trials in this study, the HGs of ostriches were collected and IgA isolation attempted using column chromatography methods. These included gelfiltration in combination with ion exchange chromatography and affinity chromatography with jacalin. These techniques produced low yields of IgA and subsequent sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) indicated fractions to contain SC and IgA heavy chains, but failed to produce intact IgA or polymeric forms in combination with SC. In addition these preparations were also contaminated with IgG. It was concluded that this source i.e. the HG and these methods were not suitable for the isolation of polymeric ostrich IgA.

Although Katz *et al.* (1974) found no antigenic differences between serum IgA (monomeric) and secretory IgA (dimeric), we felt that for the purpose of assessing the mucosal immunity in ostriches, it was imperative to isolate polymeric IgA. Since polymeric IgA in combination with SC (secretory IgA) predominates in secretions, using secondary antibodies to ostrich IgA heavy chain alone, might underestimate the actual levels of secretory IgA due to the possibility of SC preventing these antibodies from binding to the IgA heavy chain. Secondary antibodies to SC, on the other hand, may overestimate the presence of secretory IgA due to its binding to free SC. Consequently it was decided that we needed to isolate intact polymeric ostrich IgA attached to SC. HG samples were not used for this purpose as these were shown on SDS-PAGE to be contaminated with IgG. Instead tracheal secretions

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obtained from an ostrich chick that died were used. This sample was shown on SDS-PAGE to be free of IgG and contained both monomeric and polymeric forms of IgA of high molecular mass. It was therefore decided to attempt the isolation of polymeric ostrich IgA directly from gel slices of tracheal secretion proteins separated by non-reducing SDS-PAGE.

3.4. CHARACTERIZATION OF MATERNAL AND MUCOSAL IMMUNITY TO NDV IN OSTRICH CHICKS

3.4.1. CONTRIBUTIONS OF CO-AUTHORS

The following manuscript contains the original and independent work of the author of this dissertation. The author performed all experimental work, except for the preparation of the acid-treated naked bacteria from *Salmonella minnesota* R595 and the immunization of the rabbits. The contribution of each of the co-authors was as follows:

Dr. A.J. Morley was the former research veterinarian (now retired) at Klein Karoo Co-operative Research and Diagnostic Laboratory, Oudtshoorn and was responsible for overseeing the logistics of the maternal antibody trial as well as the collection of relevant samples.

Dr. A. Olivier is currently the research veterinarian at the Klein Karoo Co-operative Research and Diagnostic Laboratory, Oudtshoorn and was involved in the logistics surrounding the mucosal antibody vaccination trial as well as the collection of relevant samples.

Dr. W.P. Burger is the chief manager of research and development at the Klein Karoo Co-operative, Oudtshoorn. He supervised all collaborative research projects and was also responsible for the funding of this project.

Prof. D.U. Bellstedt is an associate professor of the Department of Biochemistry at the University of Stellenbosch, and the promoter of this dissertation. He was responsible for all collaborations with the Klein Karoo Co-operative, and was involved in all aspects of development and execution of this study.

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CHARACTERIZATION OF MATERNAL AND MUCOSAL IMMUNITY TO NDV IN OSTRICH CHICKS

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ABSTRACT

Knowledge regarding the role that immune mechanisms play in the protection of ostrich chicks against Newcastle disease virus (NDV) will be valuable in establishing effective vaccination regimes and thus in the control of NDV in ostriches. In chickens, effective control of NDV in chicks can be achieved by both passive (maternal) and active immunization. In this study, ostrich hen serum immunoglobulin G (IgG) and yolk IgY were isolated and characterized, and the transfer of maternal anti-NDV antibodies to the egg yolk determined using an enzyme-linked immunosorbent assay (ELISA). To this end, serum samples were collected from vaccinated and non-vaccinated ostrich hens and tested for anti-NDV antibodies. At the same time one egg was collected from each of these ostrich hens and the yolk tested for anti-NDV antibodies. Results indicated that anti-NDV antibodies were successfully transferred from the ostrich hen to the egg yolk. Local application of a live or inactivated NDV vaccine in ostriches has been postulated to stimulate the production of mucosal antibody and thus IgA. In this study, ostrich IgA was isolated, characterized and secondary rabbit anti-ostrich IgA antibodies produced for use in an anti-NDV ELISA. Trachea swabs were collected on a weekly basis after vaccination with a live La Sota vaccine, and tested for the presence of anti-NDV IgA antibodies. Results indicated that mucosal application of live La Sota vaccine stimulates mucosal IgA production against NDV.

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INTRODUCTION

An increase in ostrich meat production and export and therefore more intensive farming conditions in South Africa necessitated the monitoring and surveillance of endemic diseases such as ND. ND in ostriches not only poses a threat to animal industries in countries importing ostrich products, but an outbreak of ND in ostriches can potentially have a devastating effect on South African poultry trade at an international level (Huchzermeyer, 1997). Along with good management and biosecurity practices, effective vaccination of ostriches can prevent ND infection and spread. Previous studies on the efficacy of vaccination schedules currently used in South Africa, have indicated that ostrich chicks vaccinated at 5 weeks of age do not produce detectable serum antibody levels to NDV with either live or inactivated vaccines (Blignaut *et al.*, 2001). It was thus decided to re-evaluate the effectiveness of NDV vaccination in ostrich chicks.

In chickens, effective control of NDV in chicks can be achieved by both active and passive immunization (Giambrone and Closser, 1990; Sharma, 1999). Passive immunity to NDV is transferred from the chicken hen to the chick via the egg yolk and therefore, effective vaccination of chicken hens can produce a better and more extended passive immunity in their progeny (Van Eck, 1990a). Madeiros (1997) reported the protection of ostrich chicks by maternal antibodies, although the specific transfer of these antibodies has not been documented.

Active immunization on the other hand, plays an important role in cases where either the passive or active immune status of chicks is uncertain (Alexander, 1994). Due to the primary site of NDV infection being the respiratory tract, emphasis has been placed on the local application of NDV vaccines by eye drop or aerosol methods. Local application of vaccine results in the production of a local mucosal immunity consisting primarily of IgA. The advantage of local application of vaccine is that its ability to produce an immune response is not influenced by the presence of maternal antibodies. The primary indicator of the efficacy of such local vaccinations has always been serum antibody levels despite the lack of correlation between serum antibody levels and resistance to mucosal infections (Ewert *et al.*, 1979). The absence of resistance in the respiratory tract can facilitate the multiplication and subsequent dissemination of NDV, thus assisting in the spread of the disease (Holmes, 1979). Similar to mammals, chickens also possess a mucosal immune

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system of which IgA is the major immunoglobulin. Local application of a live or inactivated vaccine to NDV has been found to stimulate the production of mucosal antibody and thus IgA (Russel, 1993; Jayawardane and Spradbrow, 1995). However, no major studies on the mucosal immune response, i.e. IgA levels, to NDV vaccination have been conducted in either chickens or ostriches.

Knowledge regarding the role that immune mechanisms play in the protection of ostrich chicks against NDV will not only be valuable during epidemiological studies, but also in the control of NDV during an outbreak in ostriches. The first aim of this study was therefore to establish whether maternal immunity against NDV was transferred from the ostrich hen to the egg yolk using a previously developed anti-NDV ELISA (Blignaut *et al.*, 2001). This ELISA can be used to detect serum antibody levels to NDV and it thus had to be established whether this ELISA could detect yolk IgY levels. To this end, ostrich serum IgG and yolk IgY were isolated and characterized to establish whether they were identical. Once this was established, yolk antibody levels could be detected with this ELISA. The second aim was to establish whether mucosal vaccination of ostrich chicks with live La Sota vaccine was capable of inducing mucosal immunity in the form of measurable IgA levels using an ELISA. To this end, ostrich IgA was isolated and characterized, secondary anti-ostrich IgA antibodies produced, and an anti-NDV ELISA for the detection of mucosal IgA developed.

MATERIALS AND METHODS

Animals and Control Sera

Ostrich breeding hens used for the evaluation of passive immunity to NDV were housed on the experimental farm of the Department of Agriculture, Western Cape Province, Oudtshoorn, South Africa and on a privately owned ostrich farm in the Oudtshoorn district. Ostrich chicks used for the evaluation of mucosal immunity were housed on the experimental farm of the Department of Agriculture, Western Cape Province, Oudtshoorn, South Africa.

Control sera, used in the NDV-ELISA, were obtained from adult ostriches housed at the Regional Veterinary Laboratory, Department of Agriculture, Stellenbosch, Western Cape Province, South Africa.

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The rabbits used for immunization purposes were of mixed stock and obtained from the breeding colony of the Department of Biochemistry, University of Stellenbosch, South Africa.

Isolation of ostrich and chicken egg yolk IgY

Polyethylene glycol – Alcohol (PEG-Alc) Precipitation:

IgY was purified from ostrich and chicken egg yolk using the method of Polson *et al.* (1985). The chicken egg yolk was separated from the white (albumen) and washed with distilled water to remove as much of the albumen as possible. The yolk sac was then punctured and the yolk released into a measuring cylinder. Due to the size of the ostrich egg, an appropriate volume of yolk was extracted from the yolk sac using a 20 ml syringe.

Phosphate buffered saline (PBS; 0.15 M, pH 7.6) was added to the egg yolk in a 4:1 ratio (v/v). Pulverized PEG 6 000 was added to a final concentration of 3.5% (w/v) and stirred slowly until dissolved. The mixture was incubated for 20 min at room temperature (RT) after which it was centrifuged (5 000 x g, 20 min, RT). The supernatant was filtered through a funnel containing a loose plug of absorbent cotton wool. IgY was precipitated from the resulting filtrate by adding PEG to a final concentration of 8.5% (w/v) and stirring until dissolved. The mixture was incubated for 10 min at RT and centrifuged for 25 min at 5 000 x g. The pellet was reconstituted in PBS to a volume of 2.5 times the original yolk volume. PEG was again added to a final concentration of 12% (w/v), stirred until dissolved, incubated for 10 min at RT and centrifuged for 25 min at 5 000 x g. The pellet was reconstituted in a quarter of the original yolk volume of PBS and cooled to 0°C. To this, precooled (-20°C) 50% ethanol (v/v) was added and centrifuged in a precooled rotor for 25 min at 10 000 x g. The pellet was finally dissolved in a quarter volume (of original yolk volume) of PBS and dialysed against PBS (0.15 M, pH 7.6) for 24 h at 4°C. The dialysate was stored at 4°C until used.

Isolation of ostrich and chicken serum IgG

Ammonium Sulphate Precipitation:

Both ostrich and chicken serum was diluted with PBS (0.15 M, pH 7.2) and the Ig precipitated by the addition of an equal volume of saturated ammonium sulphate

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solution at 4°C. After centrifugation at 27 200 x g for 20 min the supernatant was removed and the precipitate dissolved in an amount of PBS equivalent to the original serum volume. This precipitation step was then repeated. The final precipitate was dissolved in an amount of PBS equivalent to the original serum volume and dialyzed against several changes of 0.005 M phosphate buffer (pH 8.0).

Ion Exchange Chromatography:

The PEG-Alc isolated IgY fractions were used as starting material for further purification of ostrich and chicken yolk IgY. The ammonium sulphate precipitated Ig fractions were used as starting material for further purification of ostrich and chicken serum IgG. The samples were first dialyzed overnight at 4°C against the starting buffer and then applied to the column. All separations were carried out on a DEAE-Sepharose CL-6B (weak anion exchanger, Pharmacia) column (10 x 2 cm x $cm.h^{-1}$); using a flow rate of 1.8 ml/min. The DEAE Sepharose CL-6B column was equilibrated with 0.005 M phosphate buffer (pH 8.0) and the same buffer used as starting buffer for elution. The relevant Ig fractions were subsequently eluted from the column with a salt gradient (0 – 1 M NaCl). Fractions (5 ml) were collected and their protein content monitored spectrophotometrically at 280 nm using a MSE Spectroplus detector.

Protein concentration determination of ion exchange fractions:

The Bradford method (1976) was modified for use directly in microtitre plates, to allow the simultaneous determination of multiple samples. Ion exchange fractions (5 μl) as well as protein standards were pipetted in duplicate into a flat-bottomed 96-well microtiter plate. To each of these, 250 μl Bradford reagent was added and the plate incubated for 5 min at RT. Absorbance was read at 620 nm with a Titertek Multiscan spectrophotometer. Bovine serum albumin (BSA, Fraction V, Miles Laboratories (Pty) Ltd, 2 mg/ml in 0.005 M phosphate buffer, pH 8.0) was used for the determination of a standard curve of absorbance against protein concentration (mg/ml). The total protein content of each fraction was calculated from the standard curve by on-line computer.

In the original Bradford protocol (Bradford, 1976) the absorbance of protein-dye complexes were measured at 595 nm. It was found that the standard 620 nm filter of the Titertek Multiscan spectrophotometer could effectively be used in the Bradford protein determination because the samples still showed about 98% of the

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absorbance at 620 nm and a linear relationship exists between protein concentration and absorbance values.

Characterization of ostrich and chicken IgG and IgY

SDS-PAGE of ion exchange fractions:

Proteins separated by ion exchange chromatography were characterized by Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) under reducing and non-reducing conditions using a Hoefer SE 200 Mighty Small™ Vertical Gel Electrophoresis Unit. The gels for the reducing SDS-PAGE consisted of a 7 cm x 8 cm x 1.5 mm resolving gel (12% T, 2.7% Cbis, 0.1% SDS, 0.375 M Tris-HCl, pH 8.8), ten well stacking gel (4.5% T, 2.7% Cbis, 0.1% SDS, 0.125 M Tris-HCl, pH 6.8) and a single tank buffer (250 mM Tris-base, 192 mM glycine, 0.1% SDS, pH 8.3). Rainbow™ coloured protein molecular weight markers (Amersham) were used as protein standards (14.3 – 220 kDa). The same procedure was used for the non-reducing SDS-PAGE, except that a 8% resolving gel was used.

Samples (22.5 µl) were mixed with an equal volume of reducing treatment buffer (0.125 M Tris-HCl, 4% w/v SDS, 20% v/v glycerol, 10% v/v 2-mercaptoethanol, pH 6.8) and 0.1 volumes of bromophenol blue (0.1% w/v in a 1.5 mM NaOH solution). Parallel to this, samples were mixed with an equal volume of non-reducing treatment buffer (0.125 M Tris-HCl, 4% w/v SDS, 20% v/v glycerol, 100 mM iodoacetamide, pH 6.8) and 0.1 volumes of bromophenol blue (0.1% w/v in a 1.5 mM NaOH solution). All samples were then placed in a boiling water bath for 2 min and put on ice until used.

Forty microlitre samples were loaded onto the SDS-PAGE gels and run at 18 mA until the bromophenol blue front reached the bottom of the gel. After electrophoresis, protein bands were fixed and stained overnight with Coomassie stain (0.025% w/v Coomassie Brilliant Blue R250, 40% methanol, 10% v/v acetic acid). This was followed by firstly destaining for 2 h in destain 1 (50% v/v methanol, 10% acetic acid) and secondly in destain 2 (5% v/v methanol, 7% acetic acid) until the gel background was stain free and protein bands were clearly visible.

The relative molecular weights (Mr) of protein bands were calculated by comparing their relative mobilities (Rf-values) with those of the protein standards plotted on a calibration curve (Log Mr against Rf-values). The Rf-value of a specific protein band

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was determined by division of its mobility (distance (mm) from top of resolving gel) by the mobility of the dye front.

Preparation of rabbit anti-ostrich IgG and anti-ostrich IgY antisera

Adsorption to acid-treated naked bacteria:

Acid-treated naked bacteria (NB) were prepared from *Salmonella minnesota* R595 as described (Bellstedt *et al.*, 1988). A fine suspension of NB in water (2 mg/ml) was prepared using a homogenizer with a loosely fitting Teflon plunger. The ion-exchange fractions of ostrich isolated yolk IgY and serum IgG were each mixed with the NB suspension so that the ratio by dry mass of protein to NB was 1 to 5 (Bellstedt *et al.*, 1987). These mixtures were placed in small pear-shaped flasks and taken to dryness by rotary evaporation. The formed complexes were resuspended at a concentration of 240 μg / 0.5 ml PBS (0.15 M, pH 7.2) for immunization purposes.

Immunization of rabbits:

The ostrich IgY- and IgG-NB complexes were used to immunize rabbits intravenously into the marginal ear vein (200 μg NB per immunization). Rabbits were immunized on days 1, 4, 7, 14, 18, 21, 28, 32 and 35, and blood was collected on days 1, 7, 14, 18, 21, 28, 35 and 42. These sera were subsequently used in an Ouchterlony double immunodiffusion test to determine the immunological relationship between IgG and IgY.

Ouchterlony (double immunodiffusion) test

An Ouchterlony test was carried out as described in Johnstone and Thorpe (1982). A 1% (w/v) agar solution was prepared in distilled water. The agar solution was heated until completely dissolved and 20 ml poured into a Petri dish (width 9 cm) and allowed to solidify. A gel punch was used to punch wells (0.2 cm apart) in the agar as shown in Figure 3. The plugs of agar were removed from the wells using a Pasteur pipette attached to a vacuum line.

Different antigen preparations, i.e. ostrich IgG and IgY, chicken IgG and IgY and ostrich serum IgG, were placed in the peripheral wells (30 μl/well) with rabbit anti-ostrich IgG and rabbit anti-ostrich IgY in the respective center wells (30 μl/well). By placing the different antigens in adjacent wells, their subsequent immunological relationships could be assessed. The Petri dish was closed and the lid sealed with

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Parafilm and placed upside down at 37°C overnight to allow complete development of precipitating bands.

Biotinylation of rabbit anti-ostrich IgG antibodies for use in NDV ELISA

The Ig fraction of the rabbit anti-ostrich IgG antiserum was isolated using ammonium sulphate precipitation as described by Hudson and Hay (1980). The final Ig precipitate was dissolved in a volume of PBS equivalent of the original serum volume and dialyzed overnight against several changes of Carbonate buffer (0.1 M, pH 8.3) at 4°C. The Ig concentration in the final dialysate was determined by absorption (280 nm) using BSA as standard. Carbonate buffer was then added in order to obtain an Ig concentration of 5 mg/ml. A solution (2 mg/ml) of biotinamidocaproate N-hydroxysuccinimide ester (Biotin, Sigma) in N,N-dimethylformamide (DMF) was prepared and added to the abovementioned Ig solution while stirring slowly for 2 h at RT. The Biotin-anti-ostrich IgG conjugate was dialyzed overnight against several changes of PBS (0.15 M, pH 7.2) at 4°C. Glycerol was added to the final preparation in a 1:1 ratio and stored at -20°C.

NDV-ELISA

Microtitre plates (96 well), pre-coated with NDV, were obtained as part of a ND antibody test kit from IDEXX GmbH, Wörrstadt, Germany. Serum and egg yolk samples, diluted 1:500 in the diluent buffer provided with the kit, were added to the NDV-coated plate (100 µl/well) and incubated for 1 h at 37°C. The plates were then washed three times with distilled water and twice with PBS (0.15 M, pH 7.2) containing 0.1% Tween 20 (PBS-Tween) (200 µl/well). Biotinylated rabbit anti-ostrich antibody diluted 1/100 in Casein buffer (0.5% casein, 0.15 M NaCl, 0.01 M Tris-HCl, 0.02% thiomersal, pH 7.6) containing 0.1% Tween 20 (Casein-Tween) was subsequently added (100 µl/well) and incubated for 1 h at 37°C. Avidin-horseradish-peroxidase conjugate (Zymed) diluted 1/100 in Casein-Tween was then added (100 µl/well) and incubated for 1 h at 37°C. Thereafter the plates were washed three times using PBS-Tween. Finally, the substrate (ABTS, 0.015% H_2O_2 in 0.1 M citrate buffer, pH 5, 100 µl/well) was added and the absorbance measured at 405 nm after 30 min at 37°C on a Labsystems Multiskan MS spectrophotometer.

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The titre values of serum and yolk samples were expressed in relation to a highly positive serum sample as relative titre values. The positive serum sample was obtained from an adult ostrich that had been hyperimmunized with live NDV vaccine. Inter-assay variation was monitored by the addition of control sera with anti-NDV antibody levels ranging from negative to strongly positive.

Transfer of maternal immunity

The passive transfer of anti-NDV antibodies from the ostrich hen to the egg yolk was determined in two trials. In the first trial, the anti-NDV antibody levels in ostrich hen sera (10 ostriches) as well as the anti-NDV antibody levels in their eggs (one egg per hen) were measured. These ten hens were previously vaccinated against NDV. In the second trial, the anti-NDV antibody levels in ostrich hen sera (9 ostriches) as well as the anti-NDV antibody levels in their eggs (one egg per hen) were measured. These ostriches had not previously been vaccinated against NDV.

One egg was taken from each of these hens at the end of the breeding season, and at the same time a serum sample was drawn from each of these ostriches and tested for the presence of anti-NDV antibodies using the NDV-ELISA. IgY was isolated from the yolk of each egg using PEG-Alc precipitation as previously described. The final dialysate was tested for anti-NDV antibodies using the NDV-ELISA.

Isolation of ostrich IgA

Sample preparation:

Samples used for IgA isolation included ostrich Harderian glands (HGs), sinus secretions taken within half an hour after slaughter, and trachea secretions from an ostrich chick that had died.

Ten HGs were finely chopped using a scalpel blade and divided between two centrifuge tubes. PBS (5 ml; pH 7.2) was added to each of the centrifuge tubes, stirred and left on ice for 2 h. The mixtures were centrifuged at 27 200 x g for 35 min after which the supernatant was decanted and PBS once again added to the HGs, stirred, incubated on ice for 2 h and centrifuged as before. The supernatant was decanted and along with the first supernatant filtered using a 45 µm sterile Millex®-HV filter (Millipore). Proteins contained within the filtered supernatants as well as the sinus and trachea secretions were next characterized using SDS-PAGE.

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SDS-PAGE:

SDS-PAGE was done in duplicate under nonreducing conditions (8% resolving gel) as described before except that an Omeg Scientific vertical gel electrophoresis unit was used, consisting of a 8.5 cm x 10.5 cm x 1.5 mm resolving gel. Samples were treated as described before, using non-reducing treatment buffer. After electrophoresis, protein bands on one half of the gel were fixed and stained and the molecular mass of the protein bands calculated as previously described.

Protein elution from SDS-PAGE gels:

The stained half of the gel was aligned (using Rainbow Markers as guide) with the non-stained half and the two protein bands of interest (referred to as IgA Protein 1 and Protein 2) excised from the non-stained gel. Proteins contained within these gel slices were next electrophoretically eluted using a Biometra Elucon apparatus (Omni-Science CC) and in the prosess concentrated on a semi-permeable membrane. By changing the polarity for a few minutes, the proteins on the membrane were moved back into the electrode buffer (250 mM Tris-base, 192mM glycine and 0.1% SDS, with pH 8.3), which was then collected using a pipette. Concentration of eluted proteins was determined using the Bradford method as before.

SDS-PAGE of eluted proteins:

Prior to electrophoresis, proteins were precipitated using absolute ethanol. Ethanol was added to the eluted proteins to a volume of 95% of the protein sample volume, mixed by inverting and incubated overnight at -20°C. Samples were then centrifuged at 13 000 x g for 15 min at 4°C. The supernatant was decanted and the pellet air-dried at RT to get rid of ethanol. Pellets were redissolved in 30 µl of reducing treatment buffer and 0.1 volumes of bromophenol blue. SDS-PAGE was performed in an Omeg Scientific vertical gel electrophoresis unit as before, but with a 10% resolving gel. Protein bands were stained with Coomassie Brilliant Blue and relative molecular weights determined as before.

Preparation of rabbit anti-ostrich IgA and IgA anti-NDV ELISA

Protein samples eluted from the SDS-PAGE gel (Protein 1 and Protein 2) were concentrated as before using absolute ethanol and subsequently adsorbed to NB for immunization of rabbits as described for IgG and IgY. Rabbits were immunized on days 1, 5, 9, 15, and 20. Blood was collected on days 1, 33, 72 and 81 from the

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rabbit immunized with Protein 1 and days 1, 33, 65 and 74 from the rabbit immunized with Protein 2. Immunoglobulins isolated from the rabbit anti-ostrich Protein 1 and anti-ostrich Protein 2 antiserum, collected on day 72 and day 65 respectively, were biotinylated as previously described for use in Western blot analysis as well as in an NDV ELISA for measuring ostrich IgA anti-NDV antibodies. The IgA anti-NDV ELISA was performed as described before for the IgG anti-NDV ELISA, except that mucosal samples were diluted 1/20 with the diluent buffer provided with the IDEXX kit. The titre values of mucosal samples were expressed as absorbance values at 405 nm.

Western Blot analysis

The specificity of the rabbit anti-ostrich IgA Protein 1 and Protein 2 was determined using Western blot analysis. Both non-reducing (8% resolving gel) and reducing (12% resolving gel) SDS-PAGE gels were run in duplicate as previously described. One gel was stained with Coomassie Brilliant Blue as before and the duplicate subjected to Western blot analysis. The SDS-gel was placed on top of blotting paper and a nitrocellulose membrane (0.45 µm, Schleicher and Schuell) placed on the gel ensuring uniform contact between the gel and the membrane on the one side, and the gel and blotting paper on the other. The gel and the membrane were sandwiched between blotting paper and Scotch Brite® pads, which were saturated with distilled water. Perspex plates were placed on either side of the sandwich and inserted into the transfer chamber, which was then filled with transfer buffer (0.05 M Tris-base, 0.2 M glycine and 20% (v/v) methanol, pH 8.3). The assembly was positioned in such a way that the gel faced the cathode and the nitrocellulose membrane the anode. The proteins were transferred electrophoretically, overnight (±16 h), at a constant current of 120 mA.

After completion of the transfer, the membrane was carefully removed and blocked with Casein buffer (0.05% Casein, 0.15 M NaCl, 0.01 M Tris-HCl, 0.02% thiomersal, pH 7.6) for 20 min at RT, to prevent the non-specific binding of proteins in subsequent steps. The membrane was next incubated for 1 h at 37 °C with biotinylated rabbit anti-Protein 1 antibodies diluted 1:300 with Casein buffer. Unbound antibodies were removed by three washes of 5 min each in PBS-Tween. The membrane was washed after each of the subsequent steps. Next the membrane was incubated (1 h, 37°) with goat anti-rabbit antibodies diluted 1:500 in Casein buffer. Rabbit-PAP, which was diluted 1:5 000 in Casein buffer, was subsequently

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added and the membrane incubated for 1 h at 37°C. Protein bands were visualized after 2 – 3 min as black/blue precipitates by the addition of a substrate solution (4-chloro-1-naphtol, cold methanol, PBS (pH 7.2) and H_2O_2 (34%) to the membrane. The reaction was terminated after colour development by washing the membrane with distilled water. The membrane was blotted dry and due to the light-sensitivity of the substrate reaction, products were stored between dry paper tissues in aluminium foil. The Western blot was repeated using biotinylated rabbit anti-ostrich Protein 2.

Vaccination trial

A group of 25 three-week old ostrich chicks were divided into two groups. Group 1 contained 15 birds and was vaccinated using a live La Sota vaccine. This group received a single vaccination (one drop in each eye, one drop in each nostril and one drop in the mouth), at 3-weeks of age. The second group contained 10 chicks, which served as control and was not vaccinated. Tracheal swabs were taken from all chicks on days 0, 7, 14, 21, 28 and 35. Each swab was incubated overnight in 200 µl PBS (pH 7.2) at 4°C. Swabs were removed and the eluate (diluted 1/20) tested for the presence of ostrich anti-NDV IgA antibodies using the NDV ELISA as described before, except that rabbit anti-ostrich Protein 1 was used as secondary antibody.

Statistical analysis of the data was performed using the General Linear Models (GLM) procedure in the Statistical Analysis System (SAS) Enterprise Guide (version 1.3.0.161), and a least significant difference (LSD) value calculated.

RESULTS

Isolation of ostrich and chicken IgG and IgY by PEG-Alc and ammonium sulphate precipitation

Ostrich and chicken IgG and IgY were isolated from serum and yolk samples. SDS-PAGE analysis (results not shown) indicated that these samples contained contaminating proteins and were thus further subjected to ion exchange chromatography. A typical ion exchange chromatography elution profile as obtained for ostrich IgY is shown in Figure 1. The elution profiles for ostrich IgG, chicken IgG and chicken IgY was similar to the elution profile as shown in Figure 1. The elution of IgG and IgY of both species produced three peaks, but these peaks were not well

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resolved and specifically peaks 2 and 3 showed considerable overlap. Although SDS-PAGE analysis (results not shown) indicated serum IgG and yolk IgY to be predominantly contained within the first peak, both antibodies eluted throughout most of the gradient, which is consistent with that found by Lebacq-Verheyden *et al.* (1974) for fowl immunoglobulins. The protein concentration was determined for all the fractions in peak 1 and the fraction with the highest concentration was used for further SDS-PAGE analysis.

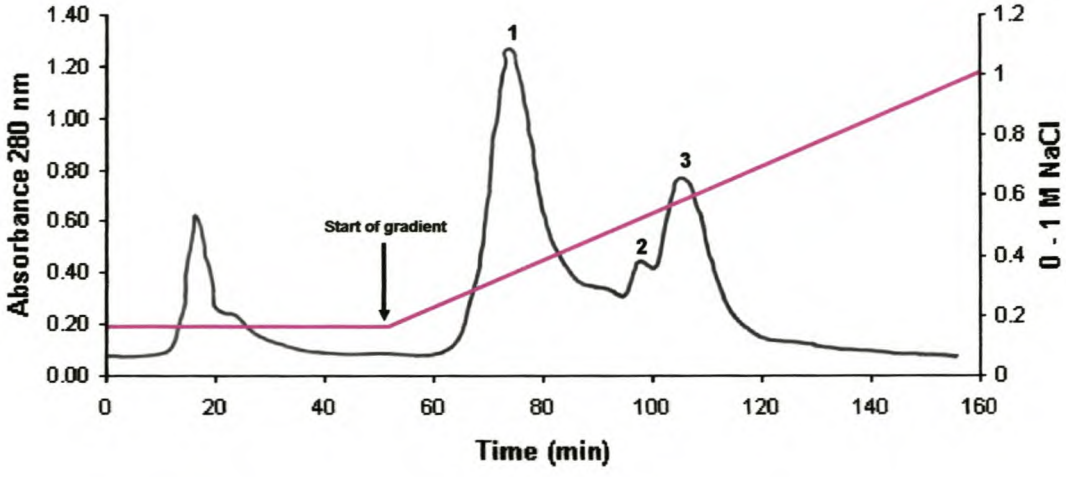

Figure 1: Separation of ostrich IgY by ion exchange chromatography. The column (10 x 2 cm, 1.8 ml/min) was equilibrated with 0.005 M phosphate buffer (pH 8.0) and proteins were eluted from the column with a 10 column volume salt gradient (0 – 1 M NaCl).

Characterization of ostrich and chicken IgG and IgY

Both reducing and non-reducing SDS-PAGE showed no apparent difference between serum IgG and yolk IgY in chickens and ostriches respectively (Figure 2A and B). Reducing SDS-PAGE (Figure 2A) shows the size of rabbit IgG heavy and light chains to be 50 kDa and 25 kDa respectively (used for reference purposes). The chicken light chain at 22 kDa, was slightly smaller than that of the ostrich light chain, 25 kDa, and their heavy chains, 65 kDa and 63 kDa for chicken and ostrich, respectively. Non-reducing SDS-PAGE (Figure 2B) showed the intact size of rabbit IgG to be 150 kDa. The intact IgG and IgY of chicken was found to be slightly larger (240 kDa) than that of the ostrich (230 kDa), but both were much larger than that of the rabbit

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(150 kDa). Lane 5 of the reducing gel (Figure 2A) showed additional higher molecular mass protein bands obove that of the chicken IgG heavy chain. These bands may be other chicken serum proteins that precipitated along with the chicken IgG during ammonium sulphate precipitation and subsequently eluted along with the chicken IgG from the ion exchange column. The absence of these bands in the yolk isolated IgY, suggests yolk to be a better source for the isolation of uncomtaminated IgG.

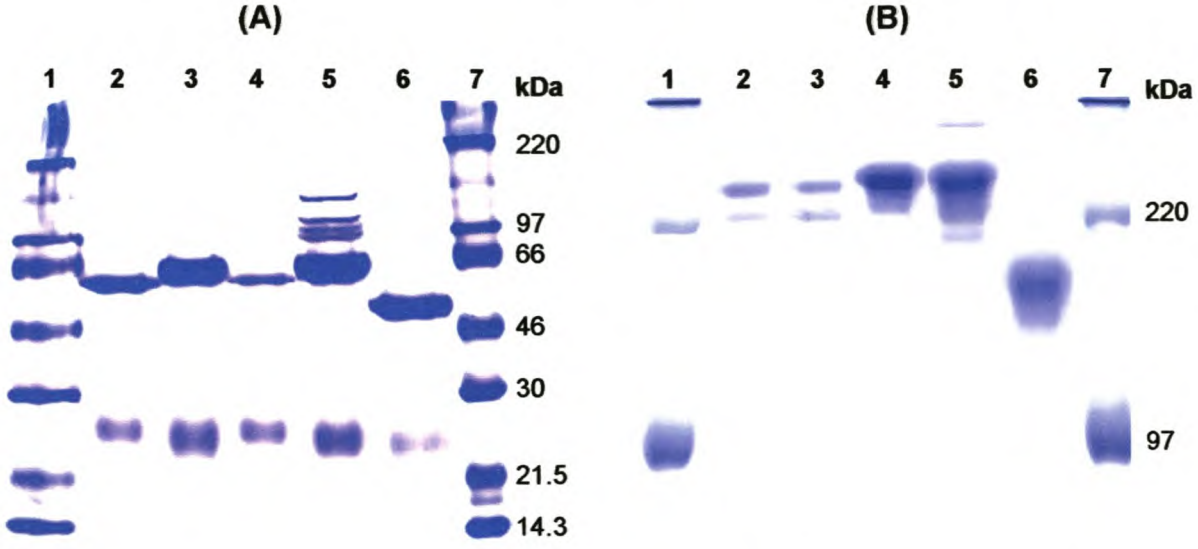

Figure 2: Protein profiles of isolated ostrich IgG and IgY on SDS-PAGE under reducing (12%) **(A)** and non-reducing (8%) **(B)** conditions. Concentrations for isolated ostrich IgG and ostrich IgY was 0.055 mg/ml and 0.191 mg/ml respectively. Concentrations for isolated chicken IgG and IgY was 0.685 mg/ml and 0.693 mg/ml respectively. Concentration for isolated rabbit IgG was 0.146 mg/ml.
(A) Lanes **1:** Rainbow™ coloured protein molecular weight markers (RM) with sizes shown in kDa; **2:** Ostrich IgY; **3:** Chicken IgY; **4:** Ostrich IgG; **5:** Chicken IgG; **6:** Rabbit IgG; **7:** RM.
(B) Lanes **1:** RM **2:** Ostrich IgY; **3:** Ostrich IgG; **4:** Chicken IgY; **5:** Chicken IgG; **6:** Rabbit IgG; **7:** RM.

Preparation of rabbit anti-ostrich IgG and IgY and use in the Ouchterlony test

The antibodies raised in rabbits to ostrich IgG and IgY recognized both ostrich serum IgG and ostrich yolk IgY in the Ouchterlony test (Figure 3). This was indicated by the precipitating line that formed between the wells containing rabbit anti-ostrich IgG or IgY, and the wells containing isolated ostrich serum IgG and yolk IgY with no cross or spur formation. This implied that ostrich serum IgG and yolk IgY was immunologically

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identical. The rabbit anti-ostrich IgG or IgY recognized neither chicken IgG nor chicken IgY.

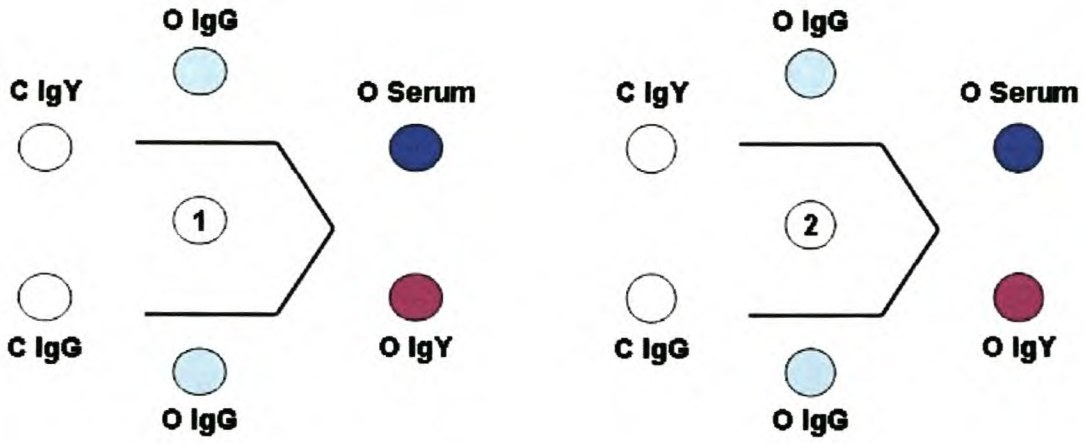

Figure 3: The immunological relationship between ostrich IgG and IgY was determined using an Ouchterlony (double immunodiffusion) test.
O = Ostrich; **C** = Chicken. Well **1** contains rabbit anti-ostrich IgG serum and well **2** contains rabbit anti-ostrich IgY. The Ouchterlony was redrawn due to poor photographic quality.

Transfer of maternal immunity

Table 1 shows the results of two trials in which the transfer of maternal antibodies (IgG) from the hen to the yolk (IgY) was investigated. In the first trial, the immunization of hens resulted in the transfer of maternal antibodies to their eggs with one exception (sample 107), where a negative IgY titre was found despite positive IgG antibody levels in the hen. Two of the immunized hens (sample 8 and 128), on the other hand, transferred anti-NDV antibodies to the yolk despite having negative antibody titres themselves. The fact that these samples were taken at the end of a breeding season could explain the negative serum antibody titres found in two of the immunized hens. Differences between hen serum antibody levels and yolk antibody levels may further be influenced by differences in time of actual egg formation, time of lay and time of collection and subsequent analysis of yolk antibody. In the second trial, unimmunized hens had low antibody titres in their eggs. Unexpectedly, three of the hens in the control group (sample 2, 6 and 7) had positive serum titres, which

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were transferred to their eggs. NDV is endemic to South Africa, which means that lentogenic strains of NDV are continually present within the environment. Positive anti-NDV titres in the unvaccinated ostrich hens may have originated from infections by such strains. One of the hens in this group (sample 3) had a negligible antibody titre in its serum, but anti-NDV antibodies were transferred to the egg yolk.

Table 1: ELISA results for the assessment of transfer of maternal antibody from ostrich hen serum (IgG) to ostrich egg yolk (IgY)

IMMUNIZED OSTRICH HENS			NON-IMMUNIZED OSTRICH HENS		
Sample No.	**IgG**	**IgY**	**Sample No.**	**IgG**	**IgY**
8	0.174	0.244	**1**	0.104	0.119
18(1)	0.320	0.482	**2**	0.341	0.587
18(2)	0.619	0.621	**3**	0.035	0.229
55	0.506	0.444	**4**	0.043	0.055
72	0.537	0.497	**5**	0.062	0.125
81	0.495	0.445	**6**	0.730	0.965
102	0.345	0.350	**7**	0.608	0.757
107	0.215	0.009	**8**	0.136	0.038
122	0.548	0.279	**9**	0.098	0.154
128	0.121	0.466	-	-	-

Serum or yolk titres with a relative titre of ≥ 0.2 are regarded as positive (Blignaut *et al.*, 2001).

Isolation and characterization of IgA

Due to contamination with IgG, HG preparations were not used for IgA isolation. Only tracheal secretions were used for IgA isolation, as SDS-PAGE analysis showed these secretions not to contain IgG (Figure 4). The protein bands that were cut out from the non-reducing SDS-PAGE gel for purification of ostrich IgA from tracheal secretions are indicated in Figure 4. The protein band above the 220 kDa molecular mass marker was referred to as Protein 1. As this protein lies beyond the range of the molecular mass markers it is possible that its size could be as high as 440 kDa, which would represent a dimeric form of IgA. The second band, which possibly

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consisted of two bands, referred to as Protein 2, was situated between the 66 and 97 kDa molecular weight markers. This band may contain secretory component (SC) and IgA heavy chain. Each of the above proteins was eluted from the non-reducing SDS-PAGE gel followed by SDS-PAGE under reducing conditions (Figure 5A and B). Both Protein 1 and Protein 2 gave a similar banding pattern with two bands of size 65 kDa and 80 kDa. The 65 kDa band of Protein 2 in Figure 5A, however, has a different mobility than that seen for Protein 2 in Figure 5B, which might be due to smiling of the gel. A light chain band (25 kDa) could only be seen in the reduced Protein 1 preparation. The 65 kDa band corresponds to that obtained by Higgins (1975) for chicken IgA heavy chain whilst Leslie and Martin (1973) obtained a protein of size 70 kDa for chicken IgA heavy chain. The 80 kDa protein band corresponds to SC of chickens (Karaca and Naqi, 1997). No protein bands could be seen for Protein 2 in Figure 5B, because of a too low protein concentration.

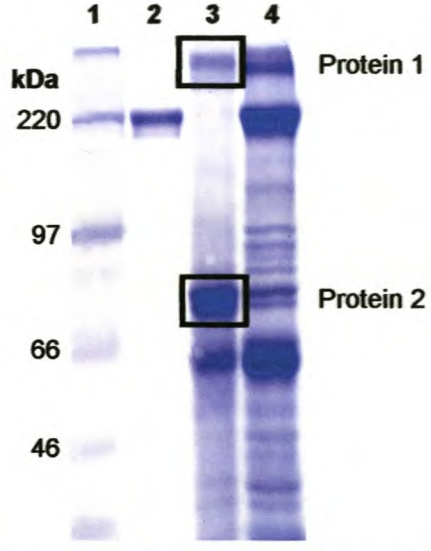

Figure 4: Protein profiles of ostrich IgG, tracheal secretions and Harderian glands separated using non-reducing SDS-PAGE (8%). The bands indicated in boxes were excised for the isolation of IgA and are referred to as Protein 1 and Protein 2. Lanes **1:** Rainbow™ coloured protein molecular weight markers with sizes shown in kDa; **2:** Ostrich IgG; **3:** Tracheal secretions obtained from an ostrich chick that had died; **4:** Ostrich HG proteins.

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Western Blot analysis

Each of the gel purified IgA proteins was used for immunizing rabbits and the resulting rabbit anti-ostrich Protein 1 and Protein 2 was used in Western blot analysis (Figure 6A, B, C and D). Rabbit anti-Protein 1 (Figure 6) detected a 80 kDa protein in the sinus secretions, Protein 1 and Protein 2 as well as higher molecular proteins in both the reducing and non-reducing gells. Halpern and Koshland (1970) found the SC of human IgA to change in molecular weight from 70 kDa to 64 kDa after reduction and alkylation, suggesting that the conformation of the SC was altered during reduction of the disulphide bonds. This could explain the difference in mobility of the 80 kDa0 protein bands in sinus secretions and Protein 1 and Protein 2. No clear protein bands could be seen in the isolated Protein 2 sample of gel A. Rabbit anti-Protein 2 (Figure 6B) detected a 80 kDa protein band in the sinus secretions and Protein 1 and Protein 2 as well as lower molecular weight protein bands. Neither rabbit anti-Protein 1 nor anti-Protein 2 detected ostrich IgG.

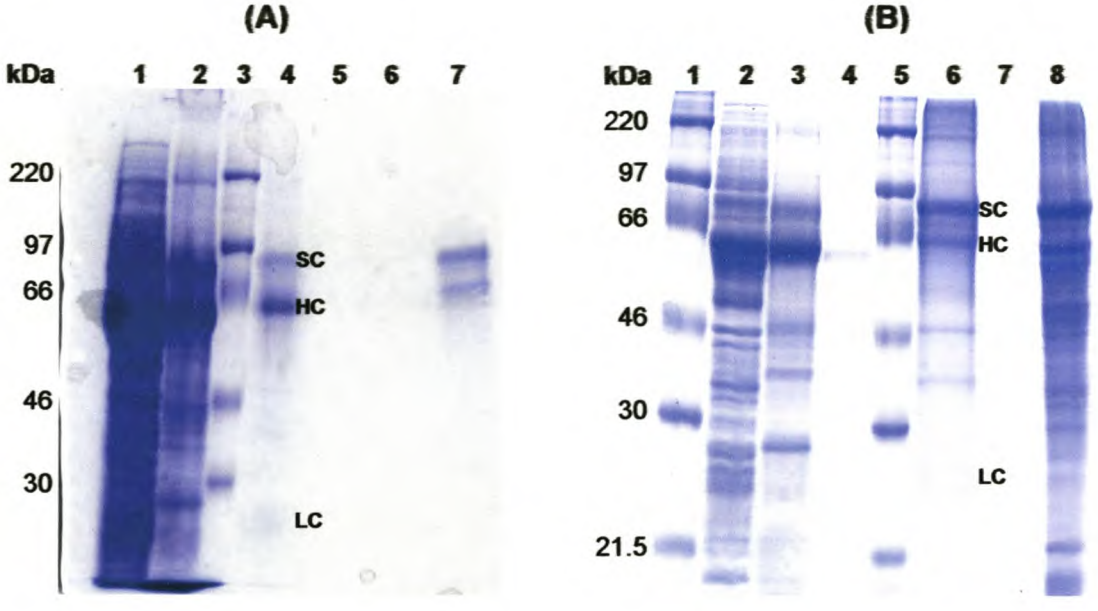

Figure 5: Protein profiles of isolated ostrich Protein 1 and Protein 2 separated using 10% **(A)** and 12% **(B)** SDS-PAGE under reducing conditions.

SC = Secretory component; **HC** = IgA heavy chain; **LC** = IgA light chain

(A) Lanes **1:** Ostrich HG preparation; **2:** Ostrich sinus secretions; **3:** Rainbow™ coloured protein molecular weight markers with sizes shown in kDa (RM); **4:** Protein 1; **7:** Protein 2.

(B) Lanes **1:** RM; **2:** Ostrich HG preparation; **3:** Ostrich sinus secretions; **4:** Ostrich IgG; **5:** RM; **6:** Protein 1; **7:** Protein 2; **8:** Ostrich tracheal secretions.

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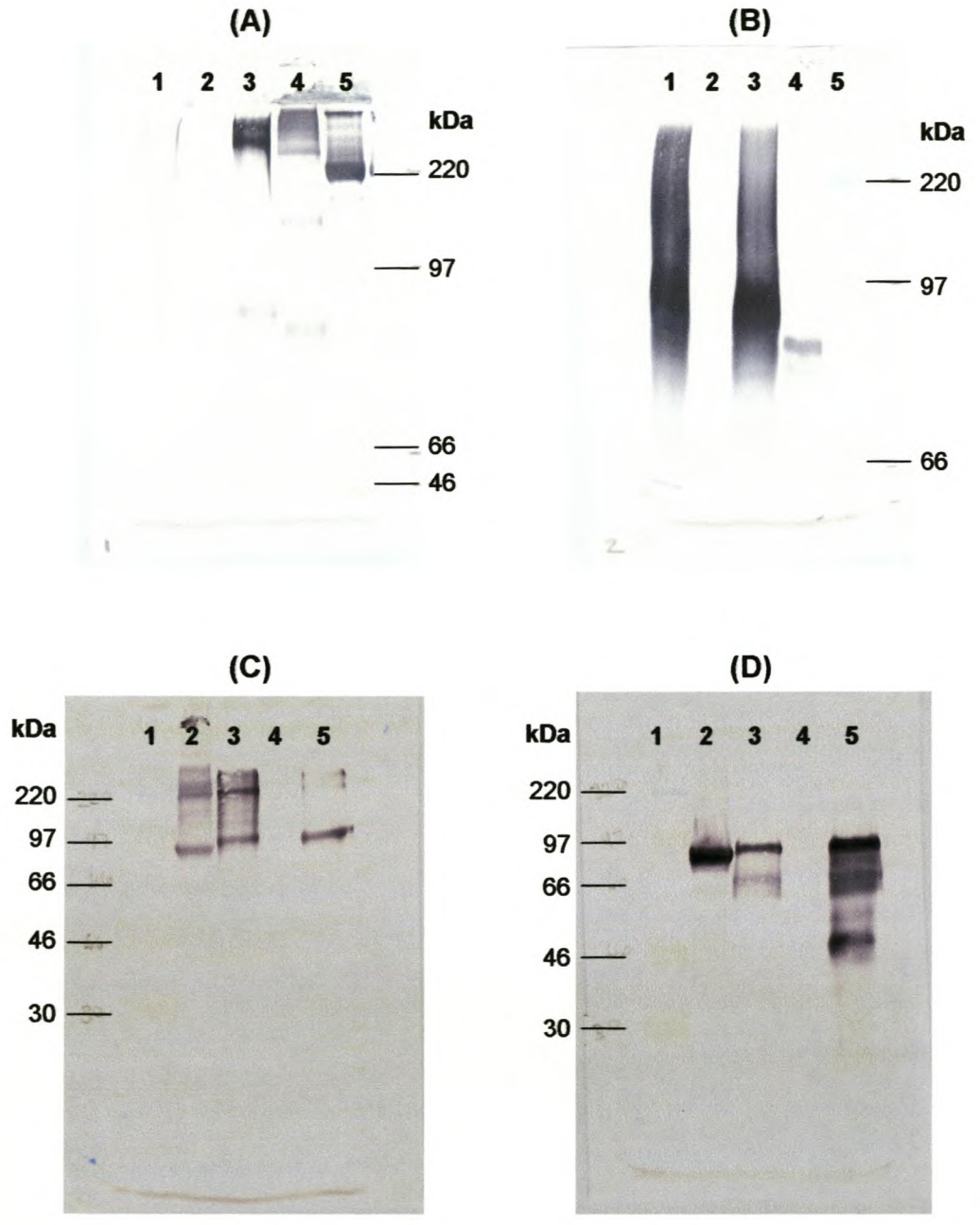

Figure 6: Western blot analysis of a 8% non-reducing gel using rabbit anti-Protein 1 **(A)** and anti-Protein 2 **(B)** and 12% reducing gel using rabbit anti-Protein 1 **(C)** and anti-Protein 2 **(D)**.

(A) Lanes **1:** Protein 2; **2:** Ostrich IgG; **3:** Protein 1; **4:** Ostrich sinus secretions; **5:** Rainbow™ coloured protein molecular weight markers with sizes shown in kDa (RM).

(B) Lanes **1:** Protein 2; **2:** Ostrich IgG; **3:** Protein 1; **4:** Ostrich sinus secretions; **5:** RM.

(C) Lanes **1:** RM; **2:** Ostrich sinus secretions; **3:** Protein 1; **4:** Ostrich IgG; **5:** Protein 2.

(D) Lanes **1:** RM; **2:** Ostrich sinus secretions; **3:** Protein 1; **4:** Ostrich IgG; **5:** Protein 2.

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IgA anti-NDV ELISA

The ability of rabbit anti-Protein 1 and anti-Protein 2 to recognise and measure mucosal IgA antibody levels in response to vaccination was tested. Anti-Protein 1 was found to produce higher ELISA antibody titres than anti-Protein 2, indicating that anti-Protein 1 preferentially recognised and detected IgA in mucosal secretions (results not shown).

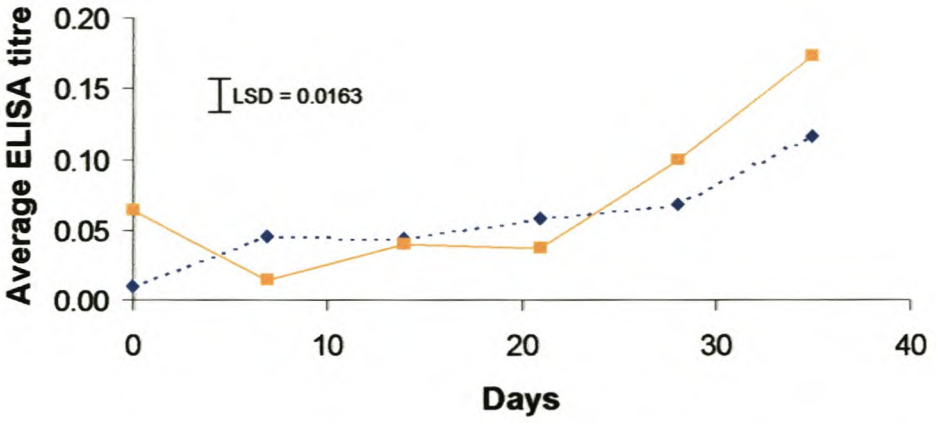

Figure 7: Mucosal immune response of ostrich chicks vaccinated against NDV with live La Sota vaccine. Group 1 (■) received a single vaccination on day 0 and group 2 (●) received no vaccine. Only group averages are indicated for each of the days.

Vaccination trial

Rabbit anti-Protein 1 was used in the subsequent trial as secondary antibody for measuring mucosal IgA in response to vaccination with a live La Sota NDV vaccine. Individual ELISA data values are given in the Appendix (page 192) and the average titre values over time in the control and vaccinated groups are shown in Figure 7. Statistical analysis showed a significant time response ($P = 0.0001$) and a significant treatment x time interaction, i.e. a significant difference between the control and the vaccinated groups over time ($P = 0.0041$). Table 2 shows the fraction and the percentage of birds that had titres ≥ 0.1 on each of the days after immunization for both the control and vaccinated groups further illustrating the differences between them. This indicated that the birds were reacting to the live La Sota vaccine and that

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the rabbit anti-Protein 1 antibodies were in fact detecting ostrich anti-NDV IgA antibodies.

Table 2: ELISA titres of ostrich chicks in the live La Sota vaccine trial with an absorbance ≥ 0.1

ELISA ABSORBANCE VALUES ≥ 0.1		
DAYS	**CONTROL**	**VACCINATED**
0	0/9 = 0%	2/14 = 14%
7	1/9 = 11%	1/14 = 7%
14	0/9 = 0%	1/14 = 7%
21	1/9 = 11%	2/13 = 15%
28	1/8 = 12.5%	4/9 = 44%
35	4/8 = 50%	8/9 = 88%

DISCUSSION

Ostrich serum IgG and yolk IgY were found to be immunologically identical which means that biotinylated rabbit anti-ostrich IgG would be able to detect both. The same anti-NDV ELISA can therefore be used to detect both ostrich serum IgG and ostrich yolk IgY. Ostrich IgG and IgY had heavy and light chains of Mr 63 000 and Mr 25 000 respectively. This is in close agreement with Cadman *et al.* (1994) who found intact ostrich IgG to have a molecular mass of 189 kDa with heavy chains of 67.5 kDa and light chains of 27 kDa.

The immunization of ostrich hens using a NDV vaccine results in serum antibody production, which is transferred to the egg yolk, as is the case in chickens (Heller *et al.*, 1977). It is recommended that ostrich breeding hens be vaccinated against NDV once a year (Huchzermeyer, 1994a). Effective vaccination of breeding hens should thus improve the immune status of ostrich chicks in the first few weeks after hatching. In this study, hens with high serum titres were reflected by high yolk antibody titres with a few exceptions. Although some authors have found a correlation between hen serum titres and yolk antibody levels, there are also reports contradicting this fact

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(Kramer and Cho, 1970; Heller *et al.*, 1977). This may be influenced by differences in time of actual egg formation, time of lay and time of collection and subsequent analysis of yolk antibody. Maternally derived immunity in the chicken will not prevent viral infection of the respiratory system of the chick, but it will reduce the pathogenic effect of the viral infection (Mockett *et al.*, 1987). Maternal immunity in chickens is, however, short lived (usually not detected beyond 5 - 6 weeks of age) necessitating vaccination of chicks (Duchatel *et al.*, 1992). Madeiros (1997) stated that maternal immunity in ostrich chicks also persists for the same period. As the present study shows that maternal anti-NDV antibodies are transmitted from the hen to the egg yolk it may be assumed that this will provide the chick with short-lived protection against viral infection.

To measure mucosal responses to NDV, IgA was isolated from sinus secretions. Two bands were identified on SDS-PAGE, which was referred to as Protein 1 and Protein 2, respectively corresponding to a dimeric and monomeric form of IgA. Reduction of Protein 1 resulted in 3 protein bands corresponding to SC (80 kDa) and IgA heavy (65 kDa) and light chain (25 kDa). Non-reducing SDS-PAGE of Protein 1 showed a 440 kDa band, which is consistent with a dimeric IgA molecule consisting of a SC, two heavy and two light chains. Although Protein 2 could not be clearly distinguished as two separate bands, it gave a similar pattern as Protein 1 after elution during gel purification and reduction (i.e. SC and IgA heavy chain), except that no band corresponding to the light chain was observed. This similarity in protein bands between Protein 1 and Protein 2 were observed after several isolation attempts. The fact that these bands were seen on a non-reducing SDS-PAGE gel might be due to degraded IgA in the original sample used for isolation. Biotinylated rabbit anti-Protein 1 was used to detect a mucosal immune response in chicks vaccinated via the respiratory route with live La Sota NDV vaccine. This further suggests that Protein 1 is in actual fact IgA.

The results obtained in the vaccination trial with live La Sota NDV, indicated that mucosal anti-NDV antibody levels only increased after day 21 at which time the chicks were 42 days old. In chickens, vaccination of day-old chicks, which possess maternal immunity results in delayed immune responses (Van Eck, 1990). Madeiros (1997) stated that maternal antibodies in ostrich chicks last approximately 45 days. As the hens of the ostrich chicks used in this trial had also been vaccinated, the

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delayed increase in mucosal antibody production may also be the result of the presence of maternal antibodies. Mucosal vaccination with live NDV vaccine strains leads to an infection of the upper respiratory tract and resultant damage to the mucosa increases capillary permeability in the trachea, which in turn causes passive transudation of serum proteins including maternal antibodies into the secretions of the respiratory tract and oral cavity (Ewert *et al.*, 1977; Kelleher *et al.*, 1988). Thus, maternal antibodies may neutralize low doses of live vaccine given by the respiratory route (Ewert *et al.*, 1977). These maternal antibodies are, however, IgG and not IgA, which is commonly associated with the mucosa. Despite this effect of maternal antibodies, they are short-lived and vaccination with live vaccine strains does eventually lead to mucosal immune responses (Zakay-Rones and Levy, 1973). This could explain the delay observed in this study. However, Ewert *et al.* (1977) also found that detection of antibody in tracheal washes collected 4 days after mucosal vaccination was complicated due to the presence of vaccine virus, which most likely absorbed any antibody entering the secretions. Mucosal antibody levels could thus only be detected when in excess of that which could be absorbed by viral antigen and this could also explain the delay observed in this study.

When day-old chickens that did not possess maternal immunity were immunized with live NDV vaccines, high levels of mucosal antibodies resulted shortly after vaccination and dropped rapidly thereafter, followed by a delayed serum antibody response (Parry and Aitken, 1973, Zakay-Rones et al., 1971). When the vaccination trial in this study was terminated at 5 weeks after vaccination, anti-NDV antibody levels were rising, but had not reached a plateau. This trial will therefore have to be repeated in order to document the maximum antibody levels resulting from this vaccination and challenge trials will have to be undertaken to assess the efficacy of this vaccination. Careful examination of the individual titres of each of the ostrich chicks used in the live La Sota vaccination trial, highlighted differences not seen when only looking at average titre values. Using an arbitrarily chosen cut-off value of ≥ 0.1 it appeared that a large proportion of the vaccinated ostrich chicks were reacting to the live La Sota vaccine in comparison to a much smaller proportion of the control group. An accurate cut-off value indicating protection will have to be established in future trials.

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The La Sota strain of NDV has a greater tendency to spread from bird to bird (Goldhaft, 1979; Meulemans, 1988; Shivdekar, 1995). This may have been the reason why some of the birds in the control group, which was housed next to the vaccinated group, may have become infected with the live La Sota strain and resulted in an immune response in these chicks. This finding should also be considered in future vaccination trials and when establishing control measures against ND as vaccination with live La Sota vaccine can contribute to the development of NDV carriers (Clubb, 1986; Alexander, 1995). NDV carriers harbouring the La Sota strain have been identified amongst slaughter ostriches in the Oudtshoorn district (Olivier, personal communication).

The availability of the anti-ostrich IgA detection antibodies produced in this study will facilitate future evaluation of mucosal immunity to NDV and subsequent development of optimal vaccination schedules in ostrich chicks. This will have to include different dosages and different NDV vaccine strains in the presence or absence of maternal immunity. Additionally, the immune responses should be followed over longer periods in order to document the duration and period of maximal IgA production.

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CHAPTER 4:
Avian Mycoplasmas

4.1. MYCOPLASMAS

4.1.1. HISTORY AND CLASSIFICATION

After the first successful cultivation of a mycoplasma by E. Nocord and E.R. Roux in 1898, there has since probably been no other group of bacteria that have caused so much confusion and controversy as to their identity and taxonomic status (Razin, 1992a; Kleven, 1997a). Initially mycoplasmas were considered to be viruses as they passed through filters blocking the passage of bacteria. This idea was abandoned with the elucidation of the true nature of viruses in the 1930s. Later on, mycoplasmas were thought to be no more than stable L-phase variants of common bacteria. This controversy only came to an end in the late 1960s when genomic analysis ruled out any relationship of mycoplasmas to stable forms of present-day walled bacteria (Razin, 1992a; Razin, 1992b). In the 1960s and 1970s, advancements in the knowledge of the ultrastructure, cell membrane, genome and metabolic pathways of mycoplasmas led to the recognition that mycoplasmas were the smallest and simplest self-replicating organisms (Razin, 1987). These findings subsequently led to the question of the place of mycoplasmas in the evolutionary scheme.

Initially two models had been formulated for mycoplasma evolution. The first model proposed by Neimark and London (1982), presumes that mycoplasmas had arisen by degenerate evolution. Mycoplasmas would have arisen after evolution and diversification of the bacteria, resulting in different mycoplasmas originating from different branches of the bacterial phylogenetic tree. Different mycoplasma species would therefore not be phylogenetically related.

The second model, proposed by Morowitz and Wallace (Morowitz and Wallace, 1973; Wallace and Morowitz, 1973), presumes that mycoplasmas arose very early in the evolution of living forms on Earth and ancestral mycoplasmas were precursors of bacteria. The small genome size of mycoplasmas was postulated to be a primordial genome that duplicated several times to produce ancestral bacteria, which would then have diverged to the present-day bacterial groups.

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Molecular evolution studies showed neither of these models to be entirely correct. Instead, with the use of rRNA sequences as phylogenetic markers, Woese (1987) showed the mycoplasma phylogenetic tree to be monophyletic. The class *Mollicutes* evolved by degenerate evolution from the *Clostridium – Lactobacillus – Streptococcal* branch of the phylum harbouring Gram-positive bacteria with a low G + C content in their genomes, characterized by rapid evolution and decreasing genetic and physiological complexity.

Accumulating molecular biology data in recent years only strengthened this view of mycoplasmas as being a clearly defined group of eubacteria. Although they are phylogenetically related to Gram-positive bacteria they retain their unique position as the smallest self-replicating prokaryotes devoid of cell walls, thus warranting their classification apart from normal bacteria as the class *Mollicutes* (Razin, 1992a).

The name *Mollicutes* is derived from the Latin *mollis* (soft) and *cutis* (skin) to denote the lack of a rigid cell wall for these organisms. *Mollicutes* is currently the only class within the division *Tenericutes* (wall-less bacteria) of the kingdom Procaryotae (Murray, 1984). The current taxonomic scheme for the class *Mollicutes*, together with the properties distinguishing the currently established taxa, are presented in Table 4.1.

In this dissertation, the trivial name *Mollicutes* will be used to denote any species included in the class *Mollicutes*, whereas the trivial names of the individual genera, e.g. mycoplasma, will be used when reference is made specifically to members of the corresponding genus.

4.1.2. MORPHOLOGY AND MOLECULAR CHARACTERISTICS

Mycoplasmas have no cell wall and are only bound by a cholesterol-containing cell membrane. The cells are pleomorphic and vary in shape from spherical, slightly ovoid or pear-shaped (0.3 – 0.8 μm in diameter) to slender branched filaments of uniform diameter (from a few to 150 μm in length). Although they are phylogenetically related to Gram-positive bacteria, mycoplasma stain negative in the Gram test. Some mycoplasmas have been reported to be chemotactic and capable of gliding on semisolid surfaces, although they are usually non-motile. Mycoplasmas are polar cells having a so-called 'tip structure' at the leading end in the form of a short, dense rodlet by which they attach themselves to host cell surfaces.

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Table 4.1: Taxonomy and molecular characteristics of *Mollicutes*

Classification	Genome size (kbp)	Mol% G +C of DNA	Hosts
Order I: *Mycoplasmatales*			
Family I: *Mycoplasmataceae*			
Genus I: *Mycoplasma*	580 - 1350	23 – 40	Humans, animals
Genus II: *Ureaplasma*	760 - 1170	27 – 30	Humans, animals
Order II: *Entomoplasmatales*			
Family I: *Entomoplasmataceae*			
Genus I: *Entomoplasma*	790 – 1140	27 – 29	Insects, plants
Genus II: *Mesoplasma*	870 - 1100	27 – 30	Insects, plants
Family II: *Spiroplasmataceae*			
Genus I: *Spiroplasma*	780 - 2220	24 – 31	Insects, plants
Order III: *Acholeplasmatales*			
Family I: *Acholeplasmataceae*			
Genus I: *Acholeplasma*	1500 - 1650	26 – 36	Animals, plants, insects
Order IV: *Anaeroplasmatales*			
Family I: *Anaeroplasmataceae*			
Genus I: *Anaeroplasma*	1500 - 1600	29 – 34	Bovine/ovine rumen
Genus II: *Asteroleplasma*	1500	40	Bovine/ovine rumen
Phytoplasma[#]	640 - 1185	23 – 29	Plants, insects

[#] Not yet characterized

Table adapted from Razin (1998)

These organized attachment structures only seem to be well developed in many of the pathogenic *Mollicutes*. Those *Mollicutes* considered as being commensals, also possess mechanisms for cell adherence, but without a well-defined tip structure or established adhesin proteins. Cytoadherence in these *Mollicutes* appear to involve specific glycoplipid and/or glycoprotein receptors on the eukaryotic host cell surface (Razin and Jacobs, 1992). A well-defined internal cytoskeleton enables the

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mycoplasma to maintain its shape in the absence of a rigid cell wall and is believed to also play a role in gliding, cell division and control of the distribution of adhesin proteins (Razin and Freundt, 1984; Razin, 1998; Trachtenberg, 1998).

Mollicutes are built of the minimum number of organelles for growth and reproduction: a circular double-stranded genome, ribosomes upon which cell proteins are assembled and a plasma membrane separating the cytoplasm from the external environment (Razin, 1992b). The smallest genomes of self-replicating, free-living organisms are found among the *Mollicutes* and range from 600 to over 2 200 kb, with overlapping values between different *Mollicutes* genera. The mycoplasma genome sizes range from 80 kb for *M. genitalium* to 1380 kb for *M. mycoides* subsp. *mycoides* (Bové, 1993; Razin, 1998).

Mycoplasmas have a characteristically low G plus C base composition. With a few exceptions, the G + C content of mycoplasma genomes range from 24 – 33 mol%. This G + C distribution along the genome is, however, uneven (Tully and Razin, 1996; Razin, 1998).

4.1.3. *IN VITRO* CULTURE

The extreme simplicity of the mycoplasma genome, and thus the cells, is made possible by adopting a parasitic mode of life. Living in the protective environment of their host as well as depending on their host for essentially the complete spectrum of amino acids, fatty acids, cholesterol and vitamins obviates the need for a cell membrane and many biosynthetic systems. Thus, difficulty in imitating the nutritional milieu provided by the host has complicated the successful *in vitro* cultivation of many mycoplasmas (Razin, 1992b).

Mycoplasma species from avian sources generally require a protein-rich medium with an additional 10 – 15% animal serum, but further supplementation with a yeast-derived component may be beneficial (Kleven, 1997a). They are facultatively anaerobic, but some are obligate anaerobes that are killed by exposure to minute quantities of oxygen. Mycoplasmas use either sugars or arginine as major energy source and require cholesterol or related sterols for growth. The mycoplasmas tend to grow slowly, preferring temperatures of 37 - 38°C and are rather resistant to thallium acetate and penicillin, which are frequently employed in media to retard the growth of contaminant bacteria and fungi (Razin and Freundt, 1984). Avian

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mycoplasma colonies start forming on agar media after 3 - 10 days at 37°C, however, some species may develop colonies within one day (Kleven, 1997a).

The typical colony of mycoplasmas, under adequate growth conditions, has a characteristic 'fried egg' appearance. Variation in colony morphology has been described, but cannot be used to differentiate the various species (Razin and Freundt, 1984; Kleven, 1997a).

4.1.4. DISTRIBUTION

Mollicutes are widespread in nature as parasites, commensals, saprophytes or pathogens of humans, domestic and wild mammals as well as birds, reptiles, fish, arthropods and plants (Razin and Freundt, 1984; Tully, 1996a; Razin, 1998). Mycoplasmas are parasites and pathogens of a wide range of mammalian and avian hosts (Razin and Freundt, 1984). They usually exhibit a rather strict host, organ and tissue specificity that most likely reflects their parasitic mode of life and nutrionally exacting nature (Kleven, 1997a; Razin, 1998). There are, however, numerous examples of mycoplasmas present in hosts and tissues different from their normal habitats (Razin, 1992a). Strains of *M. arginini*, *M. canis* and many established *Acholeplasma* species have been found to occur in a wide variety of different animal hosts (Tully, 1996a). There have also been reports on transmission of animal mycoplasmas to humans and *vice versa* (Erickson *et al.*, 1988; Tully, 1993).

The primary habitats of human and animal mycoplasmas are the mucous surfaces of the respiratory and urogenital tracks, the eyes, alimentary canal, mammary glands and joints (Kleven, 1997a; Razin, 1998). Mycoplasmas in humans and animals are mostly surface (extracellular) parasites rarely invading tissues. After cytoadherence (a prerequisite for colonization and infection) some mycoplasmas have been shown to enter the host cell, either by penetration through their specialized tip structure (*M. penetrans* and *M. genitalium*) or by internalisation (*M. fermetans* and *M. hominis*). Once the mycoplasma is internalised in the cytoplasm, it may be free or inside a vesicle. By entering the host cell, mycoplasmas become isolated from host mycoplasmacidal immune mechanisms and selective drug therapies, which in turn promote the establishment of a latent or chronic infection state (Razin, 1992b; Razin, 1998).

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4.1.5. PATHOGENICITY

Mycoplasmas are close to the 'ideal parasite', usually living in harmony with their host. In the case of pathogenic mycoplasmas, infections are rarely of the fulminant type and rather follow a chronic course, but the molecular basis of mycoplasma pathogenicity remains largely unknown (Razin, 1992b).

The chronicity of mycoplasma infections raises the question as to how these wall-less, rather fragile organisms resist the immune defence mechanisms of their host. First, this is achieved by the generation of a versatile surface coat by high frequency phenotypic switching, involving variable antigens (Razin, 1998). This allows the mycoplasma to avoid the immune system and subsequent antibody attack (Tryon and Baseman, 1992). Second, there is a complex network of mycoplasma induced specific and non-specific immune reactions between the mycoplasma and the host immune system (Razin, 1998).

The mycoplasma-induced specific protective defence mechanisms are elicited by invading mycoplasmas and include the production of local as well as systemic anti-mycoplasmal antibodies, stimulation of cell-mediated immunity and opsonization and phagocytosis of the organism. These specific reactions are essential for resistance and protection against mycoplasma infections, but have also been shown to play a role in the development of lesions as well as the exacerbation of mycoplasma-induced diseases.

The mycoplasma-induced non-specific immunomodulatory effects on the immune system of the host include: inducing either suppression or polyclonal stimulation of B and T lymphocytes; inducing cytokine production; increasing cytotoxicity of macrophages, natural killer cells and T-cells; enhancing the expression of cell receptors and complement activation. This potential of mycoplasmas to either stimulate or suppress the host immune system contributes to their pathogenic properties. It also enables them to evade or suppress the host defence mechanisms, thus establishing a chronic, persistent infection (Razin, 1992b; Razin, 1998). The clinical symptoms seen in humans and animals are therefore more suggestive of damage due to the immune and inflammatory responses of the host itself, rather than to the direct toxic effects of mycoplasma cell components (Razin, 1998).

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4.2. MYCOPLASMA INFECTIONS OF DOMESTIC POULTRY

Avian mycoplasmas have been studied mainly in association with domestic poultry (Jordan, 1979). Several species of mycoplasma have been isolated from other avian sources, but their pathogenicity has not been critically researched (Jordan, 1979; Jordan, 1996; Kleven and Levisohn, 1996).

In domestic poultry there are four major pathogenic mycoplasma species: *M. gallisepticum*, *M. synoviae*, *M. iowae* and *M. meleagridis*. These species are considered economically important, not only because of their association with disease and therefore costly control programs, but also due to their association with reduced production, reduced hatchability, downgrading of carcasses and sub-optimal grading of chicks and poults (turkey chicks) (Jordan, 1979; Ley and Yoder, 1997).

The following overview of mycoplasma infection, diagnosis and control in poultry is given with reference to the above four mycoplasmas.

4.2.1. EPIDEMIOLOGY

4.2.1.1. Natural host

M. gallisepticum infection occurs naturally in chickens and turkeys, commonly resulting in disease of the respiratory tract (Ley and Yoder, 1997). In chickens and turkeys it causes tracheitis and airsacculitis and in turkeys additional sinusitis (Jordan, 1979; Ross, 1993). In South Africa, *M. gallisepticum* was isolated from turkeys and free-ranging chickens (Buys *et al.*, 1989; Thekisoe *et al.*, 2003).

Chickens, turkeys and guineafowl are the natural hosts of *M. synoviae*, which causes airsacculitis and synovitis (Jordan, 1979; Ross, 1993; Kleven, 1997b). In South Africa, Buys (1976) reported the isolation of *M. synoviae* from chickens showing symptoms of synovitis and arthritis as well as cases of airsacculitis.

The natural host of *M. iowae* is the turkey, but isolation from chickens is not uncommon. *M. iowae* is generally associated with reduced hatchability and embryo mortality in turkeys. Experimentally it has been shown to induce embryo mortality in chickens and airsacculitis in chickens and turkeys (Ross, 1993; Jordan, 1996; Kleven and Baxter-Jones, 1997). No reports of *M. iowae* infections in South Africa could be found.

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M. meleagridis is a specific pathogen of turkeys, causing airsacculitis in turkey poults and can have an immunosuppressive effect in young stock (Ross, 1993; Jordan, 1996; Yamamoto and Ghazikhanian, 1997). Buys *et al.* (1989) reported the isolation of *M. meleagridis* from turkeys in South Africa.

4.2.1.2. Incubation

Although incubation periods have experimentally been determined for the different mycoplasma species, it is difficult to define meaningful incubation periods under natural conditions, as there are many variable factors that influence the onset and extent of clinical infection (Ley and Yoder, 1997).

The association of a mycoplasma species with disease is influenced by the pathogenicity of the specific infecting strain (Jordan, 1996) and factors that might influence this variability in pathogenicity include virulence, tropism, number of organisms (dosage), and route of infection (Jordan, 1979). Features of the host, on the other hand, that might influence the onset and extent of clinical disease include species, genetic constitution, age (embryo, neonate or mature bird), immune status, intercurrent infection with other microorganisms and non-living debilitating factors (Jordan, 1979). Non-living debilitating factors known to exacerbate mycoplasma infections are trauma, temperature, humidity, ventilation, excess atmospheric ammonia, dust, nutritional deficiency and social stress associated with intensive management. Disease severity is known to increase during winter months and in some instances, to affect younger birds more severely than mature birds (Jordan, 1979; Jordan, 1996; Ley and Yoder, 1997; Kleven, 1998).

Mycoplasma infections can additionally be aggravated by single or multiple interactions with other infectious agents such as *Escherichia coli* (*E. coli*), NDV, Infectious bronchitis virus (IBV), *Haemophilus paragallinarum*, laryngotracheitis virus, adenovirus and reoviruses (Kleven, 1997b; Ley and Yoder, 1997; Kleven, 1998). There have also been reports of synergism between different mycoplasma species such as *M. gallisepticum* and *M. synoviae* or *M. meleagridis* and *M. synoviae* (Kleven, 1998).

4.2.1.3. Transmission

Transmission of *M. gallisepticum* may occur by direct contact of susceptible birds with infected carrier chickens or turkeys. Lateral spread may occur by contaminated

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airborne droplets, dust as well as feathers and equipment. Infection may also spread via venereal and egg transmission (Jordan, 1979; Ross, 1993; Ley and Yoder, 1997).

The spread of *M. synoviae* appears to be similar to that of *M. gallisepticum*, spreading mainly through contact and droplet particles from the respiratory tract as well as egg transmission (Jordan, 1979; Ross, 1993; Kleven, 1997b).

M. iowae is transmitted through the egg which probably becomes infected in the oviduct. The oviduct may become infected by venereal transmission, signifying the role of the male in disease transmission. The spread of infection may also be potentiated by unhygienic technique during artificial insemination (Jordan, 1996; Kleven and Baxter-Jones, 1997).

M. meleagridis is primarily transmitted through the egg, which as in the case of *M. iowae* infections, become infected in the oviduct indicating a possible role of venereal transmission. Indirect transmission can result from management practices including sexing, vaginal palpation, artificial insemination and vaccination during which mycoplasmas may be manually carried from infected to non-infected turkeys via contaminated hands, clothing or equipment. Airborne transmission may occur among newly hatched poults, but is of little significance once a bird has reached sexual maturity (Ross, 1993; Jordan, 1996; Yamamoto and Ghazikhanian, 1997).

Persistence of mycoplasma infections inside the host may vary from a few weeks to months, years and even life (Jordan, 1996; Ley and Yoder, 1997). Outside the body of the host, however, the mycoplasma organism is fragile and its survival may be limited to a few days or less under usual poultry house conditions. If the organism is protected by exudates and/or a cold environment, survival may be prolonged (Jordan, 1996). A study done by Christensen *et al.* (1994) indicated that *M. gallisepticum* and *M. synoviae* could potentially survive for several days on materials such as feathers, human hair and cotton clothing. *M. iowae* on the other hand is also fragile outside the body, but survives longer and is more resistant to the common mycoplasmacidal drugs than other avian pathogenic mycoplasmas (Jordan, 1996).

4.2.2. CLINICAL SIGNS AND LESIONS

M. gallisepticum infections frequently cause no clinical signs or mortality except in the very young, but the additional presence of other organisms or debilitating factors can

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cause complications with resulting exacerbation of signs (Jordan, 1979; Jordan, 1996). Characteristic signs in adult chicken flocks are coryza, tracheal rales, nasal discharge, coughing and sneezing. Other signs include weight loss, decline in egg production as well as keratoconjuctivitis. In turkeys there is a typical swelling of the paranasal sinuses from sinusitis preceded by nasal discharge with foaming of eye secretions. In the presence of tracheitis or airsacculitis there are signs of tracheal rales, coughing and laboured breathing. Breeding flocks may have a drop in egg production (Jordan, 1979; Jordan, 1996; Ley and Yoder, 1997). Gross lesions may be so mild as to be imperceptible or consist only of catarrhal exudates in nasal and paranasal passages as well as in the trachea, bronchi, lungs and air sacs. At a later stage caseous exudates may appear in the air sacs or attached to the air sac walls. In severe cases of air sac infections in chickens there may be pericarditis, perihepatitis, as well as massive airsacculitis. Arthritis and synovitis, especially of the hocks, have been seen in chickens and turkeys. In the case of keratoconjunctivitis there is marked oedema in the facial subcutis and eyelids (Jordan, 1979; Jordan, 1996; Ley and Yoder, 1997).

During *M. synoviae* infections, clinical disease is the exception rather than the rule, but when clinical signs do occur, they may take either a respiratory or arthritic form. In chickens the respiratory form may consist of mild rales and coryza, but conditions can be exacerbated if infection is associated with viruses, bacteria and other predisposing factors. In turkeys no respiratory signs are usually observed although *M. synoviae* has been isolated from sinus exudates. In chickens and turkeys the acute arthritic form involves depression, pallor of the face and comb, lameness, retarded growth, swelling around joints and breast blisters, however, swelling of the joints are not so prominent in turkeys. Birds become listless, dehydrated, emaciated and a greenish discoloration of droppings is frequently seen (Jordan, 1979; Jordan, 1996; Kleven, 1997b). Gross lesions during the early stages of infection consist of viscous creamy to grey exudates involving the synovial membranes of the tendon sheaths, joints and keel bursae. Later on a caseous exudate may be found in the tendon sheaths, joints and even extending into muscle and air sacs. Gross lesions are usually not seen in the upper respiratory tract, but airsacculitis may be present in the respiratory form of the disease. Other lesions include an enlarged spleen, swollen, mottled and pale kidneys, swollen and mottled liver with a green or dark red

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colour and atrophied thymus and bursa of Fabricius (Jordan, 1979; Jordan, 1996; Kleven, 1997b).

M. iowae infections cause no clinical signs in mature turkeys, except for reduced hatchability of eggs (usually 2 - 10%). Gross lesions of affected embryos consist primarily of stunting and congestion with various degrees of hepatitis, oedema, splenomegaly and occasionally a down abnormality. These lesions, however, cannot be considered pathognomonic and may be similar to lesions observed when embryos are overheated in the incubator. Experimental infections with *M. iowae* cause mild to moderate airsacculitis in chickens and turkeys. Inoculation of day-old poults with a virulent strain of *M. iowae*, results in stunting, poor feathering, tenosynovitis and leg abnormalities such as chondrodystrophy, rotated tibia, toe deviations and occasionally erosion of articular cartilage of the hock joint and rupture of the digital flexor tendon. Similar lesions may be observed in experimental chicks including rupture of the digital flexor tendon, but lesions are usually less severe than in turkeys. Under field conditions such severe lesions are rarely seen, perhaps because infected embryos do not hatch (Jordan, 1996; Kleven and Baxter-Jones, 1997).

M. meleagridis commonly occurs as a silent infection in adult birds causing no clinical signs. Even in poults originating from infected dams, the high rate of airsacculitis rarely produces respiratory signs, but infected poults may show signs of poor growth together with skeletal abnormalities and abduction of the wing feathers. *M. meleagridis* acts synergistically with *M. iowae* in producing severe airsacculitis and with *M. synoviae* resulting in sinusitis (Jordan, 1996; Yamamoto and Ghazikhanian, 1997). Skeletal lesions include osteodystrophy with shortening, bending and twisting of the tarsometatarsal bones as well as abnormalities of the cervical vertebrae. There have also been reports of synovitis and oedema (Jordan, 1996; Yamamoto and Ghazikhanian, 1997).

4.2.3. DIAGNOSIS

The respiratory and other signs as well as postmortem lesions described for the different mycoplasmas are not pathognomonic for any given mycoplasma infection. The signs and lesions are therefore no more than suggestive of mycopasma presence. As a result, the diagnosis of mycoplasma infections depends either on isolation and identification of the organism, or an examination of sera (Jordan, 1979).

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4.2.3.1. Diagnostic samples

Samples for *M. gallisepticum* can be taken from live birds, fresh carcasses, carcasses frozen when fresh, dead-in-shell embryos, chicks or poults. Swabs from live and dead birds can be taken from the trachea, choanal cleft, oropharynx, oesophagus, cloaca and phallus. Exudates may be aspirated from the infraorbital sinuses, joints, trachea, airsac, turbinates and lungs. These swabs and exudates may be inoculated directly to mycoplasma broth or agar medium (Jordan, 1996; Ley and Yoder, 1997).

M. synoviae can be recovered in acutely infected birds from the trachea, lungs, air sacs, synovial, bursal or joint lesions. In chronically infected birds isolations from the upper respiratory tract are more reliable, however, organisms may no longer be viable making the isolation of organisms in the chronic stages of infection difficult or even impossible (Jordan, 1979; Jordan, 1996; Kleven, 1997b).

M. iowae can be isolated from the oviduct, cloaca, phallus and semen of adult chickens and turkeys, however, isolations become less frequent with age. After experimental infection, *M. iowae* can be isolated from the gastrointestinal tract, cloaca, oropharynx and airsacs of recently hatched stock. *M. iowae* is also present in high numbers in dead embryos (Jordan, 1996; Kleven and Baxter-Jones, 1997).

M. meleagridis can be isolated from the respiratory tract, airsacs, cloaca, bursa of Fabricius, infraorbital sinus and occasionally from hock deformities in young poults and from the cloaca, oviduct and semen of breeding birds (Jordan, 1979; Jordan, 1996; Yamamoto and Ghazikhanian, 1997).

4.2.3.2. Isolation and identification

The different above-mentioned samples can be used to inoculate a suitable solid agar or broth medium of choice, but no single medium formulation is sufficient to support the growth of all mycoplasma species (Kleven and Levisohn, 1996). The fastidious nature of this organism is exemplified in the rather complex growth requirements of different strains and the fact that from time to time, different batches of the same media may not equally support growth of the organism (Yamamoto and Ghazikhanian, 1997).

Mycoplasma isolates can be identified using one of the following techniques:

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- Preparation of antigens from isolates and testing them against known antiserum. This method is, however, rarely satisfactory with recently isolated cultures (Ley and Yoder, 1997).
- Mycoplasma free chickens or turkeys can be inoculated with the isolate and their serum tested with known mycoplasma antiserum. This is referred to as an *in vivo* bioassay (Jordan, 1996; Ley and Yoder, 1997).
- Direct or indirect immunofluorescence using mycoplasma colonies on the surface of agar plates. This is a rapid and reliable method that can be used with mixed cultures to identify the different *Mollicute* species, however, closely related organisms may cross-react (Jordan, 1996; Kleven and Levisohn, 1996; Ley and Yoder, 1997).
- Agar gel precipitin test (Ley and Yoder, 1997).
- The direct immunoperoxidase test (Ley and Yoder, 1997).
- Comparison of protein banding patterns resulting from SDS-PAGE (Khan *et al.*, 1987; Ley and Yoder, 1997).
- Immunoblotting allows individual proteins (mycoplasma antigens or adhesins) in a complex mixture to be detected and analysed (Thirkell and Precious, 1996; Tully, 1996b; Ley and Yoder, 1997).
- Restriction fragment length polymorphisms (RFLP) of DNA, which have greater sensitivity than SDS-PAGE for differentiating strains of a single species from one another (Garcia *et al.*, 1995; Ley and Yoder, 1997).
- DNA and rRNA gene probes. Despite the high sensitivity of this technique when used as a diagnostic system, it is still not sensitive enough for many clinical applications (Johansson, 1996; Ley and Yoder, 1997).
- PCR using specific DNA nucleotide sequences (Jordan, 1996; Ley and Yoder, 1997).

RFLP, DNA probes and PCR are especially useful for epidemiological investigations during outbreaks or for identification and differentiation of vaccine strains from natural infections. It has the additional potential of being able to directly demonstrate specific

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DNA sequences under conditions where culture methods may not succeed (Kleven and Levisohn, 1996).

4.2.3.3. Serology

Serological tests for demonstration of specific antibody are useful for flock monitoring in control programs and to aid diagnosis when infection is suspected (Ley and Yoder, 1997). Serological tests that can be used are as follows:

- Serum plate agglutination (SPA). This test is quick, relatively inexpensive and highly sensitive. It is, however, used only as an initial screening test for flock monitoring and serodiagnosis due to non-specific reactors in some flocks infected with *M. synoviae* or those recently vaccinated against various agents with either oil-emulsion vaccines or vaccines of tissue culture origin (Ross, 1993; Kleven and Levisohn, 1996; Ley and Yoder, 1997).
- Haemagglutination inhibition (HI) test. This test is time consuming, reagents are not commercially available and the test is not very sensitive. In the case of *M. gallisepticum* infections, some isolates fail to agglutinate red blood cells and some do not stimulate the production of HI antibody. It is therefore used mainly as a confirmatory test for sera reacting positively to the SPA test (Jordan, 1979; Ross, 1993; Jordan, 1996; Ley and Yoder, 1997).
- ELISA test. The ELISA test is more sensitive and specific than either the SPA or HI test and is used increasingly as initial screening test for flock monitoring and serodiagnosis (Ross, 1993; Brown *et al.*, 1996).

In instances where symptoms are not specific for a mycoplasma infection, the application of both culture and serological test procedures may be necessary to differentiate a mycoplasma infection from other infecting agents (Ley and Yoder, 1997).

4.2.4. PREVENTION AND CONTROL

Excluding infection is the most satisfactory means of control and where spread of infection can be limited, it is to be advocated (Jordan, 1996). Since the pathogenic mycoplasmas are egg-transmitted, control is initiated in the breeding stock and comprises the following (Jordan, 1979; Jordan, 1996):

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i) Treatment of hatching eggs to reduce transmission of mycoplasma through the embryo and progeny. This can be done by treatment of eggs with anti-microbial drugs or by heating or both. These two methods greatly reduce, but cannot completely eliminate the possibility of egg transmission (Ley and Yoder, 1997).

ii) Keeping progeny flocks in relatively small groups (200 - 300 for chicks and less than 50 - 100 for poults).

iii) Groups should be regularly monitored for infection and if any member of a group shows evidence of infection, the whole group must be eradicated.

iv) Effective biosecurity measures should be taken i.e. management of groups in such a way as to avoid the entry of infection.

The economic benefit of having a specific pathogen-free status in the breeding stock flows on to their progeny (McMartin, 1994). Having a pathogen-free status, however, depends on the following conditions (Whithear, 1996):

i) The mycoplasma-free flock must be placed in a clean environment.

ii) Possibility of introduced infection must be eliminated by appropriate isolation and/or biosecurity.

The first condition can often not be met due to the economic imperative of running multiple-age flocks on a single site as is the case in the layer industry and also on breeder farms. The second condition is also difficult to satisfy because of pathogenic mycoplasmas that are capable of surviving outside their host for longer than expected together with the range of air-borne spread that is greater than previously believed. There is thus a serious risk of cross-infection between sites given that poultry raising tends to be concentrated in localised areas (McMartin, 1994).

Although biosecurity is the preferred method of control when trying to exclude an infection from stock, immunization or anti-microbial medication may be required in instances where infection cannot be reliably excluded by economically sustainable biosecurity (Whithear, 1996).

Antibiotic treatment is of value in the face of clinical disease and for egg treatment to reduce infection. Resistance of certain mycoplasmas, such as *M. synoviae*, to anti-

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microbials (Jordan, 1996), along with the high cost of anti-microbial therapy has shifted the attention to the development of effective vaccines against pathogenic mycoplasmas.

At present commercially manufactured vaccines are either killed whole cells (bacterins) or living cultures. Bacterins contain inactivated organisms suspended in either aqueous oil emulsion or aluminium hydroxide adjuvants. Bacterins have the advantage that they are non-infectious and therefore pose no risk of cross-infection to other stock or reversion to virulence. They are, however, expensive both in terms of requiring large amounts of antigen and the need for handling birds individually during administration (Whithear, 1996).

Live mycoplasma vaccines contain either naturally occurring strains of moderate virulence or artificially attenuated strains that stimulate long-term protective immunity by multiplying in the bird. Completely avirulent strains do not appear to stimulate the immune system as effectively. An important quality of any live vaccine is that it should provide immunity without the ability to cause disease or spread to other susceptible birds. This delicate balance may be complicated due to the ability of certain mycoplasma species to interact synergistically with other infectious agents. These synergistic interactions can produce even more severe infections if birds are subjected to environmental and/or physiological stress (Whithear, 1996).

4.3. MYCOPLASMA INFECTIONS OF OSTRICHES

Scientific knowledge of mycoplasma-associated diseases in ostriches is either fragmented or incomplete and technical aspects of diagnostic and/or screening tests mostly absent (Verwoerd, 2000).

4.3.1. EPIDEMIOLOGY

Mycoplasmas in ostriches are usually associated with respiratory diseases and generally cause rhino-tracheitis, which is an inflammation of the upper respiratory tract, and airsacculitis, a common extension of nasal infections. An infectious form of rhino-tracheitis, commonly referred to as 'snotsiekte' by farmers, is generally found in feedlot birds and is brought on by cold, windy weather in the winter as well as extreme heat conditions in the summer (Allwright, 1995; Huchzermeyer, 1994b).

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Age groups that are mostly susceptible to mycoplasma infections are usually 4 – 11 month old feedlot birds exposed to inefficient management practices. However, mycoplasmas can even be isolated from three-week-old chicks with symptoms and lesions. On their own, mycoplasma infections are usually not lethal, although the presence of a pathogenic strain is suspected in the Oudtshoorn and Calitzdorp district that is especially lethal to chicks of 3 – 4 months of age. The influence of mycoplasmas on egg production and subsequent chick quality is suspected but uncertain (Olivier, personal communication).

As is the case in poultry, mycoplasma infections can be brought about, or existing infections exacerbated by stress, environmental conditions and concurrent bacterial or viral infections (Allwright, 1995). Environmental conditions include: high levels of ammonia, dust, temperature and ventilation (Allwright, 1995). In the Oudtshoorn area, mycoplasmas tend to be a problem especially during seasonal changes i.e. autumn and spring (Olivier, personal communication). Opportunistic bacteria that are usually isolated include: *E. coli*, *Pseudomonas spp.*, *Pasteurella spp.*, *Klebsiella spp.*, *Bordetella spp.* and *Staphylococcus aureus*. These bacteria not only exacerbate symptoms of existing infections but also together with mycoplasmas, are especially lethal for 3 – 7 month old chicks (Allwright, 1995; Olivier, personal communication). Concomitant viral infections include: influenza A and ND. Usually symptoms are indicative of viral presence such as green urine in the case of influenza and swollen heads together with nervous symptoms in the case of ND (Allwright, 1995).

Morbidity in some flocks may be as high as 30 – 70% but mortality is variable and depends on opportunistic infections as well as the ability of chicks to keep on eating to help build up resistance. During winter months this situation is complicated due to increased metabolic demands for the maintenance of body temperature (Olivier, personal communication).

4.3.2. SIGNS AND LESIONS

Signs include upper respiratory infections such as sinusitis, rhinitis (nasal discharge), foaming conjunctivae, tracheitis, tracheal rales due to sinusitis (post nasal drip), coughing, laryngitis, slime in the throat and air sac infections. Ostriches with typical signs of sinusitis and foaming conjunctivae are shown in Figure 4.1 A and B. Air sac infections are not clinically visible although labored breathing may suggest its

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presence. There is usually no loss in appetite but weight gain may be poor (Allwright, 1995; Huchzermeyer, 1994b; Olivier, personal communication).

Figure 4.1: An illustration of mycoplasma infections in ostriches: **(A)** Arrow indicates swelling of the paranasal sinuses due to sinusitis. This is usually preceded by a nasal discharge and foaming of eye secretions. **(B)** Arrow indicates foaming of the eye secretions.

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Gross lesions in recently infected birds include: light rhinitis or sinusitis with thick clear mucous in the sinuses and trachea. Air sacs have a wet appearance with visible veins and exudates inside the air sacs forming a clear to white foam. In older or complicated (other bacteria or viruses) infections, air sacs may be dark red with thickened air sac walls and filled with exudate. Sinuses may be swollen and filled with a thick yellow-white exudate (Olivier, personal communication).

4.3.3. DIAGNOSIS

At the Klein Karoo Research and Diagnostic Laboratory in Oudtshoorn mycoplasma infections have, to date, been diagnosed by *in vitro* cultivation. The preferred samples for mycoplasma isolation are tracheal swabs, although air sac, sinus or conjunctival swabs can also be used. The swabs are usually plated directly onto suitable agar medium, but broth medium can also be used. In addition to the fact that mycoplasmas are difficult to cultivate, it is not possible to identify different mycoplasmas species just on the basis of culture morphology. Initial attempts were made to identify the cultivated mycoplasmas using the Fluorescent Antibody Test (FAT) that was performed at a laboratory in Liverpool (England). Due to the expense involved and their inability to identify any of the mycoplasma cultures, further testing was discontinued (Peyrot, personal communication.). One of the mycoplasma cultures was, however, identified by the FAT as *M. synoviae*. Subsequent DNA isolation and sequencing of the mycoplasma 16S rRNA gene did not verify this result, but indicated it to be a unique mycoplasma. Due to possible cross-reactivity, the identification of mycoplasma species on the basis of antigenic differences is therefore unreliable.

4.3.4. CONTROL

As is the case with poultry, excluding infection is the most satisfactory means of control. Given the apparent susceptibility of ostriches to poultry mycoplasmas and the lack of information on subsequent pathological conditions, it is recommended that ostrich farms are kept free of any poultry (Verwoerd, 2000).

In poultry, control is initiated in the breeding stock and comprises amongst other things the eradication of a whole group or flock if any member shows evidence of infection. Due to the high value of individual ostriches, this method of control is not economically justifiable. At present, however, breeding pairs are mainly kept under

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extensive conditions and they are not exposed to production stress under instensive conditions, as is the case with feedlot birds. They also have the advantage of a low level of exposure to other diseased birds as well as other stress factors such as direct cold, wind or dust (Olivier, personal communication).

For the treatment of mycoplasma infections, broad-range antibiotics are used (Allwright, 1995), but no registered mycoplasma vaccine for ostriches is currently available.

4.3.5. OSTRICH SPECIFIC MYCOPLASMAS

It was reported that *M. gallisepticum* and *M. synoviae* were isolated regularly in winter from feedlot ostriches kept at the Onderstepoort Veterinary Institute, Pretoria, South Africa with respiratory symptoms concomitant with other opportunistic pathogens (Verwoerd, 2000). The method of identification was, however, not stated in this report and it must be assumed that it was based on the morphology of isolated mycoplasmas. As this method of identification is often unreliable, these identifications may be questionable. In addition, commercial poultry was kept in close proximity to these ostriches and cross-infection with poultry mycoplasmas could not be excluded.

A serological survey was undertaken in ostriches in northern Italy using the rapid plate test with specific antigen for *M. gallisepticum*, *M. synoviae* and *M. meleagridis*. Some of the ostrich sera showed positive reactions to *M. gallisepticum*, *M. synoviae* and *M. meleagridis*. All the seropositive ostriches had, however, been kept in paddocks together with backyard poultry, which showed clinical symptoms and laboratory findings of mycoplasma infection (Peccati *et al.*, 1996). Experimental infection of ostriches with *M. gallisepticum* and *M. synoviae* resulted in colonization of *M. gallisepticum* in the tracheas of young ostriches, with *M. synoviae* giving inconclusive results (Cline *et al.*, 1997).

Consequently, the isolation of poultry mycoplasmas from ostriches raises the following question: Do only poultry mycoplasmas infect ostriches, or are they also being infected with ostrich specific mycoplasmas? In a study done by Shivaprasad (1993) on neonatal mortality in ostriches, mycoplasmas were isolated from 32 out of 372 ostriches submitted. Most isolations were made from the lung or trachea of ostriches with no clinical signs, respiratory problems or any lesions involving the lung or trachea. None of these 32 isolates were *M. gallisepticum, M. synoviae, M. iowae*

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or *M. meleagridis*. The strain was, however, not identified. In a report on factors influencing the survival of ostrich chicks (Smith, 1993), mycoplasmas were implicated in respiratory infections and subsequently isolated from ocular and tracheal swabs. These ostriches had no gross or histological lesions typical for mycoplasmosis in other birds. In this instance, culturing was found to be more effective than serology for detecting mycoplasma in ostriches and the mycoplasma that was isolated, was not one of the four major poultry mycoplasmas, but an unknown species.

As stated by Peccati *et al.* (1996), there is an urgent need to identify mycoplasma species of interest in ostriches and to evaluate their infectivity and pathogenicity with subsequent determination of diagnostic methods. The present study is specifically aimed at the identification of ostrich specific mycoplasmas and this study is outlined in Chapter 5.

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CHAPTER 5:

Identification of ostrich specific mycoplasmas using 16S rRNA gene sequences

5.1. INTRODUCTION

The arduous task of identifying mycoplasmas can be simplified with the use of molecular genetic techniques such as the PCR. A commonly used phylogenetic marker gene, 16S rRNA, has successfully been used in the identification and diagnosis of different poultry mycoplasmas. Using these 16S rRNA sequences, species-specific primers for easy detection and identification of different poultry mycoplasmas were developed (Nascimento *et al.*, 1991; Lauerman *et al.*, 1993; Moalic *et al.*, 1997). Given the success of these approaches, the objectives of this study were as follows:

1) Identification of mycoplasmas that infect ostriches in the Oudtshoorn area using 16S rRNA gene sequences.
2) Sequencing of amplified 16S rRNA genes and phylogenetic comparison with known 16S rRNA sequences of other avian mycoplasmas.
3) Development of specific 16S rRNA primer pairs for each of the ostrich mycoplasmas.

The results that were obtained in achieving each of the objectives are described at the end of this chapter in the form of an independent manuscript. The manuscript is preceded by background information on certain aspects of the experimental design as well as strategies used in obtaining each of the above objectives.

5.2. IDENTIFICATION OF OSTRICH MYCOPLASMAS

The first step towards investigating the epidemiology and pathogenicity of mycoplasmas infecting ostriches is to identify the mycoplasma strain responsible for these infections. The identification and classification of mycoplasmas is hampered by the relative scarcity of useful morphological and biochemical properties. Even the use of serological tests are limited due to the frequent occurrence of common antigens

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among mycoplasmas such as *M. imitans* that cross-reacts serologically with *M. gallisepticum* (Bradbury *et al.*, 1993). These reasons have prompted the much wider use of molecular genetic techniques in mycoplasma identification and classification (Razin, 1985).

5.2.1. PCR

Since the first publication of the PCR by Saiki *et al.* (1985), it has had a major impact on molecular biology being the technique that is most widely used on a routine basis (Kidd and Ruano, 1995; White, 1996). The ability to amplify DNA sequences from clinical samples offers the potential of rapid detection and specific identification of pathogens, especially of organisms such as mycoplasmas, which are difficult to cultivate (Fredrick and Relman, 1988). In addition to its use in diagnostic microbiology, the PCR also aids in studying the epidemiology of pathogens by being a useful technique for easy molecular strain typing (White, 1996).

The PCR uses the basic elements of the natural DNA replication process by which, through an *in vitro* process, it allows the enzymatic production of large amounts of a specific DNA fragment of defined length and sequence, from small amounts of template such as genomic DNA (White 1996; McPherson and Moller, 2000). The PCR involves combining DNA template with oligonucleotide primers, deoxynucleotide triphosphates (dNTPs), a thermostable DNA polymerase and suitable buffer containing amongst others $MgCl_2$ (Saiki, 1989). Mg^{2+} ions form a soluble complex with dNTPs, which is essential for dNTP incorporation. It also stimulates the polymerase activity and increases the melting temperature (T_m) of the double-stranded DNA and primer/template interaction (Newton and Graham, 1997).

The first PCR experiments used the Klenow fragment of *E. coli* DNA polymerase I, which produced incomplete target sequences and with its low optimum temperature of 37°C was inactivated easily and had to be added to the reaction continuously (Saiki *et al.*, 1985). The introduction of thermostable Taq DNA polymerase, isolated from *Thermus aquaticus*, allowed automation of the PCR process and thereby increased the overall yield as Taq polymerase can extend sequences up to 1 000 bases in length in less than a minute (Saiki *et al.*, 1988; Arnheim and Erlich, 1992). In addition to this, Taq polymerase has a temperature optimum of 72°C, allowing primer annealing and extension to be carried out at much higher temperatures thereby

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increasing the overall stringency of the reaction and in the process minimize mismatch priming (Saiki, 1989).

A typical PCR cycle consists of three steps (Kidd and Ruano, 1995):

i) Denaturing step:
During this step, the temperature is increased (usually 94 - 95°C) causing the hydrogen bonds between the basepairs of the template DNA strands to break, forming single-stranded DNA (McPherson and Moller, 2000).

ii) Annealing step:
In this step the temperature is lowered followed by annealing of primers to their complementary DNA template sequences in such orientation that their 3'-hydroxyl ends face the target sequence that will be amplified. The T_m of the relevant primers will determine the temperature of the annealing step (McPherson and Moller, 2000).

iii) Extension step:
The temperature is once again increased (usually 72°C) whereby the DNA polymerase anchors itself to the primer-template complex and extracts free dNTPs from the medium, which are added to the 3'-hydroxyl group of the primer (McPherson and Moller, 2000). This results in extension of the primer along the template strand in the 3'-end direction, forming a new duplex molecule. In the initial cycles the extension products of each primer extends far through the target region thereby including the sequence of the other flanking primer. After the first few cycles, the major product will be a DNA fragment equal in length to the lengths of the two primers together with the intervening target DNA (Arnheim and Erlich, 1992).

In the above three steps each duplex DNA molecule produced in one cycle will in the next be denatured and each of the strands (original DNA template and newly synthesized DNA template) will act as template for further DNA synthesis. These steps are repeated until a sufficient amount of product is produced (Arnheim and Erlich, 1992).

The PCR process can be divided into three phases. The first is the screening phase, where during the early cycles, pimers search template DNA for complementary

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sequences. The second phase is the amplification phase, where during the mid cycles, primer pairs act together in amplifying the desired target DNA fragment causing the product to accumulate exponentially (Arnheim and Erlich, 1992). During this phase the previously synthesized products are preferred templates (Kidd and Ruano, 1995). The third phase is the plateau phase, where during the late cycles, amplification becomes suboptimal. This is firstly due to product accumulation whereby the active sites of the enzyme become totally occupied. Secondly, the decrease in primer to template ratio, promotes self-annealing of newly synthesized DNA strands thereby blocking primer binding to target DNA. This results in binding of primers to and amplification of non-specific target DNA. When the amount of enzyme becomes limiting or when self-annealing becomes significant, the targe DNA ceases to increase exponentially whilst non-specific target DNA in turn, starts increasing exponentially. It is thus important to choose the correct number of cycles so that the PCR reaction can be stopped before a plateau is reached and to avoid an increase in non-specific DNA product (Kidd and Ruano, 1995; McPherson and Moller, 2000).

5.2.2. 16S rRNA AS PHYLOGENETIC MARKER GENE

The criteria for choosing a gene as a useful phylogenetic marker are as follows (Sogin *et al.*, 1972; Stackebrandt and Woese, 1981; Maniloff, 1992):

i) The gene must be universally distributed, i.e. it must be present in every organism in order to be used as a universal phylogenetic measure.

ii) The gene product must have functional constancy in every organism and therefore be under the same selective pressure in every organism.

iii) The gene must not be subject to significant lateral transfer, as this would obviate its use as a phylogenetic measure.

iv) The gene base sequence must be a sufficiently slow “clock”, i.e. the primary sequence must change slowly with time in order to be a record of phylogenetic changes (random base changes) over long genealogical periods.

v) The gene must be readily isolated and sequenced for it to be used as an experimentally feasible phylogenetic marker.

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The small ribosomal subunit rRNAs meet these requirements. These molecules are universally distributed, appear to be highly constant in function and are easily isolated (Stackebrandt and Woese, 1981). Mycoplasmal ribosomes resemble typical eubacterial ribosomes in having three rRNA species that is organized in the order 16S – 23S – 5S and these genes function as an operon (Glaser *et al.* 1992; Razin, 1998).

Among the ribosomal molecules, 16S and 23S rRNA can be used to characterize prokaryotic agents, because both are large enough to contain a sufficient number of phylogenetically informative positions. Due to its larger size, 23S rRNA contains a greater level of sequence variability and therefore, generally allows greater resolution than 16S rRNA. Few taxonomic or phylogenetic studies, however, have addressed the broad-range use of the 23S rRNA gene for bacterial identification. As a result, the amount of published sequence information from the 23S rRNA gene is sparse in comparison to 16S rRNA (Kolbert and Persing, 1999).

Support for the extensive use of 16S rRNA sequences as a phylogenetic tool is given by the fact that 16S rRNA contains two genealogical clocks. Some parts of the 16S rRNA sequence have evolved relatively slowly (sequences conserved during microbial evolution) thus providing a measure of phylogenetic distances. Other parts of the sequence have evolved relatively rapidly thus measuring more recent phylogenetic events (Stackebrandt and Woese, 1981; Maniloff, 1983; Maniloff, 1992). The great weight given to 16S rRNA sequences in mycoplasma species identification as well as phylogeny and taxonomy led the *Mollicutes* Taxonomy Committee to recommend the inclusion of the 16S rRNA sequence as one of the minimum requirements in the description of any new *Mollicutes* species (Whitcomb *et al.*, 1995).

5.2.3. STRATEGY USED FOR THE SEQUENCING AND AMPLIFICATION OF THE 16S rRNA GENE

Mycoplasma containing samples were firstly identified by PCR using a 16S rRNA based general primer pair (GPO3 and MGSO) which is able to detect mycoplasma at both genus- and species level (Van Kuppeveld *et al.*, 1992). In the present study, the GPO3 primer sequence was, however, slightly changed by moving the position of the primer on the 16S rRNA sequence, two base pairs to the left. This was done to avoid the possibility of secondary structure formation of the GPO3 primer as indicated

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using the computer program Primer Designer (version 2.0). The primer was subsequently renamed GPO3F.

The amplification and sequencing strategy used for the 16S rRNA gene of mycoplasma positive samples is illustrated in the following manuscript (Figure 1). Initially, sequencing of the 16S rRNA gene was approached using three *E. coli* primers named 16F27, 16F540 and 16R1541 (Hauben *et al.*, 1997). This approach was, however, largely unsuccessful and only one mycoplasma sample could be sequenced successfully. The *E. coli* primers were next used in combination with the general mycoplasma primers. Using the primer combinations of 16F27 and MGSO as well as GPO3F and 16R1541, two overlapping sequences were generated thereby providing a sequence of the entire mycoplasma 16S rRNA gene.

5.2.4. PHYLOGENETIC ANALYSIS

16S rRNA sequences generated for each of the ostrich mycoplasmas were aligned with the 16S rRNA sequences of other known avian mycoplasmas as well as related *Mollicutes* (found in animal hosts) and bacteria. Sequences were aligned using the DNA and Protein Sequence Alignment (DAPSA) program (Harley, 1998). Using the computer program PAUP* (phylogeny analysis using parsimony), version 3.10β (Swofford, 1993), the phylogenetic relationships of the ostrich isolated mycoplasmas to these species was established by parsimony analysis. The different species used in the phylogenetic analysis is shown in Table 5.1.

The different methods used for the calculation of phylogenetic trees are based on the use of algoritms. Methods that can be used fall into two general categories (Baldauf, 2003). The first are distance-matrix methods including UPGMA, neighbour-joining and Fitch-Margoliash. The second are discrete data methods or tree searching methods including parsimony, maximum likelihood and Bayesian methods. With distance methods, the differences between sequences (percentage) are calculated and the distances assembled into a tree. The discrete method on the other hand, examines each column of the alignment separately and produces a tree that best accommodates all of this information. Depending on the data set, each of these methods may produce a similar result. In a parsimony analysis, the phylogenetic accuracy of a tree is calculated by bootstrapping. Bootstrapping is done by taking random subsamples of the relevant dataset and building a tree from each of these.

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Table 5.1: Species used in phylogenetic analysis

HOST	SPECIES	STRAIN	ACCESSION NO.	SEQUENCE LENGTH	REFERENCE
Chicken	*M. gallisepticum*	A5969	L08897	1-1519	FEMS Microbiol Lett. 128(3), 321-325, 1995
	M. gallinarum	PG16	L24105	1-1435	
	M. gallinaceum	DD	L24104	1-1431	
	M. gallopavonis	-	AF064062	1-1480 (P)	
	M. glycophilum	486 (T)	AF412981	1-1450 (P)	
	M. synoviae	WVU 1853	X52083/L07757	1-1538	Thesis: Dept. of Vet. Paraclinical Sciences, The University of Melbourne, 1990
	M. pullorum	CKK	U58504	1-1464	
	M. lipofaciens	R171 (T)	AF221115	1-1456 (P)	Int. J. Syst. Evol. Microbiol. 51(2), 633-643, 2001
	M. iners	-	AF064063	1-1492 (P)	
	U. gallorale	ATCC 43346	U62937	1-1523 (P)	Int. J. Syst. Bacteriol. 46 (4), 1183-1184, 1996
Turkey	*M. meleagridis*	17529	L24106	1-1437	
	M. gallopavonis	-	AF064062	1-1480 (P)	
	M. iowae	PPAV	U29676/X55271	1-1510	Int. J. Syst. Bacteriol. 41(4), 473-478, 1991
Duck	*M. anatis*	1340 (T)	AF412970	1-1450 (P)	
	M. imitans	4229	L24103	1-1439	
Geese	*M. anseris*	1219 (T)	AF125584	1-1461 (P)	
Pigeon	*M. columbinasale*	694 (T)	AF221112	1-1458 (P)	Int. J. Syst. Evol. Microbiol. 51(2), 633-643, 2001
	M. columbinum	MMP-1 (T)	AF221113	1-1457 (P)	Int. J. Syst. Evol. Microbiol. 51(2), 633-643, 2001
	M. columborale	MMP-4 (T)	AF412975	1-1449 (P)	
Buzzard	*M. buteonis*	BbT2g (T)	AF412971	1-1448 (P)	
Various	*M. cloacale*	383 (T)	AF125592	1-1453 (P)	
Vulture	*M. corogypsi* (Black Vulture)	BV1	L08054	1-1467 (P)	Int. J. Syst. Evol. Microbiol. 43 (3), 585-590, 1993
	M. gypis	B1/T1 (T)	AF125589	1-1462 (P)	
Falcon	*M. falconis*	H/T1	AF125591	1-1468 (P)	
Human	*U. urealyticum*	960 NCTC 10177	M23935	1-1464	J. Bacteriol. 171, 6455-6467, 1989
	C. innocuum	B-3 ATCC 14501	M23732	1-1544	J. Bacteriol. 171, 6455-6467, 1989
Other	*A. laidlawii*	JA1	M23932	1-1508	J. Bacteriol. 171, 6455-6467, 1989

(P) Partial Sequence

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The frequency with which different parts of the tree is reproduced for all the random subsamples is calculated and expressed as a percentage value. Parsimony analysis was chosen in this study, but for no specific reason.

Phylogenetic analysis of the 16S rRNA gene of ostrich isolated mycoplasmas revealed ostriches in the Oudtshoorn area to be infected by three unique ostrich mycoplasmas. Mycoplasmas are named according to their host or disease pathology. In this study, the ostrich mycoplasmas were named '*Mycoplasma struthiolus*' (Ms), from *Struthio camelus*, the species name of ostriches. The three mycoplasmas were designated Ms01, Ms02 and Ms03 respectively until formally described. Results of the phylogenetic analysis are given in the following manuscript.

5.3. PRIMER DEVELOPMENT FOR OSTRICH MYCOPLASMAS

Based on computer alignments (DAPSA) of the 16S rRNA gene sequences of Ms01, Ms02 and Ms03, primer pairs were selected for each from an area of variable sequence (Neefs *et al.*, 1990). Primers were designed using the computer software package, Primer Designer Version 2.0, applying the following criteria (McPherson and Moller, 2000; Newton and Graham, 1997):

i) Primers should be 20-30 nucleotides long, thereby providing good specificity for a unique target sequence.

ii) The primers should contain approximately equal numbers of each nucleotide.

iii) The T_m temperatures of a primer pair should preferably be within 5°C of each other.

iv) Runs of 3 or more G or C's should be avoided as this could lead to mispriming.

v) Primers must not have complementary sequences on either of the extreme 3' ends as this can lead to primer-dimer formation. Internal complementary sequences should also be avoided due to the possibility of secondary structure formation.

Using the above criteria, several primers pairs were developed for each of the three ostrich mycoplasmas (see Table 2 in the following manuscript). The different primers

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were developed with T_m temperatures as similar as possible in order to allow the development of a multiplex PCR for the simultaneous detection of Ms01, Ms02 and Ms03. The success of the development of primers for the specific detection of Ms01, Ms02 and Ms03 will be discussed in the following manuscript.

5.4. THE IDENTIFICATION AND EPIDEMIOLOGY OF THREE NOVEL OSTRICH MYCOPLASMAS

5.4.1. CONTRIBUTIONS OF CO-AUTHORS

The following manuscript contains the original and independent work of the author of this dissertation. The author performed all experimental work, except for the cultivation of the mycoplasmas which was performed at the Klein Karoo Research and Diagnostic Laboratory in Oudtshoorn and the clean-up and analysis of cycle sequencing reactions, which was performed as a routine and paid service by the University of Stellenbosch Core DNA Cycle Sequencing Facility. The contribution of each of the co-authors was as follows:

Ms. B.M. Peyrot is the veterinary technologist at the Klein Karoo Co-operative Research and Diagnostic Laboratory in Oudtshoorn and was responsible for the cultivation of all mycoplasma samples.

Dr. A. Olivier is the research veterinarian at the Klein Karoo Co-operative Research and Diagnostic Laboratory in Oudtshoorn and was involved in the development of this project as well as collection of post mortem mycoplasma samples. The field veterinarian at the Klein Karoo Co-operative Research and Diagnostic Laboratory in Oudtshoorn, Dr. B. Ganzevoort, performed sample collections on ostrich farms.

Dr. W.P. Burger is the chief manager of research and development at the Klein Karoo Co-operative, Oudtshoorn. He supervised all collaborative research projects and was also responsible for the funding of this project.

Prof. D.U. Bellstedt is an associate professor of the Department of Biochemistry at the University of Stellenbosch, and the promoter of this dissertation. He was responsible for all collaborations with the Klein Karoo Co-operative, and was involved in all aspects of development and execution of this study.

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THE IDENTIFICATION AND EPIDEMIOLOGY OF THREE NOVEL OSTRICH MYCOPLASMAS

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ABSTRACT

Mycoplasmas have been implicated in certain disease syndromes in ostriches and are usually associated with upper respiratory tract infections causing rhino-tracheitis. Mycoplasma infections are considered economically important as they cause stock losses, reduced production, reduced hatchability and downgrading of carcasses. Although poultry mycoplasmas have been shown to infect ostriches, the existence of unique ostrich specific mycoplasmas has been suggested. In this study, a large number of ostrich mycoplasmas were isolated and identified using 16S rRNA gene sequencing. These sequences indicated that ostriches in the Klein Karoo, Central Karoo and Garden Route areas of the Western and Northern Cape Provinces carry three unique mycoplasmas. Phylogenetic analysis of the 16S rRNA gene sequences of the three isolated ostrich mycoplasmas showed them to be quite divergent and fall into two distinct phylogenetic mycoplasma groupings. Unique sequences within the 16S rRNA gene of the ostrich mycoplasmas were subsequently used for the development of specific primers for the detection and diagnosis of mycoplasma infections in ostriches.

INTRODUCTION

Increased commercial ostrich production has resulted in a system of intensive rearing, which has brought certain disease syndromes to the fore. Mycoplasmas have been implicated in such disease syndromes, either as primary or secondary causes of these diseases syndromes. Mycoplasma infections are considered

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economically important as they cause stock losses, reduced production, reduced hatchability and downgrading of carcasses.

Mycoplasmas in ostriches are usually associated with respiratory disease and causes rhino-tracheitis (commonly referred to as 'snotsiekte' by farmers), which is an inflammation of the upper respiratory tract and if left untreated, leads to infection of the air sacs. Although poultry mycoplasmas have been shown to infect ostriches (Peccati *et al.*, 1996; Cline *et al.*, 1997; Verwoerd, 2000), the existence of unique ostrich specific mycoplasmas has been suggested (Shivaprasad, 1993, Smith, 1993).

The identification and classification of mycoplasmas is, however, hampered by the relative scarcity of useful morphological and biochemical characters. This has prompted the much wider use of molecular genetic techniques in mycoplasma identification and classification. The 16S rRNA gene is unique to prokaryotes and is routinely used for identification purposes. Sequence analysis of conserved genes such as the 16S rRNA gene, allows phylogenetic relationships to be established between mycoplasmas thereby making the identification of novel species possible without laboratory cultivation (Wang and Wang, 1996).

In this study, a large number of ostrich mycoplasmas were isolated and identified using 16S rRNA gene sequencing and their phylogenetic relationships determined. Unique sequences within this gene were subsequently used for the development of PCR assays for the detection and diagnosis of mycoplasma infections in ostriches.

MATERIALS AND METHODS

Sample collection and processing

A total of 3 eye, 20 sinus, 23 choana, 168 trachea, 18 air sac, 2 jejenum, 2 ileum, 1 caecum, 1 colon, 3 cloaca and 1 yolk swabs were collected from ostriches on different ostrich farms in the Northern and Western Cape Provinces, South Africa. These ostrich farms were situated in different districts of the Klein Karoo including, Ladismith, Calitzdorp, Oudtshoorn, Volmoed, Dysselsdorp, De Rust, Uniondale, Noll, Schoemanshoek, Herold, Lategansvlei as well as Victoria-West in the Central Karoo. Samples were also collected from districts within the Garden Route including Heidelberg, Riversdale, Albertinia and Mossel Bay. Samples were collected either

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from live ostriches showing symptoms of respiratory infection or sinusitis, or during post-mortem examinations. Six trachea swabs were also taken from free-range chickens that were in close contact with ostriches on farms in the Oudtshoorn district.

If immediate processing of samples was not possible, swabs were placed into Amies transport medium without charcoal and stored at 4°C for up to 24 hours. It was also found that Modified Chanock's broth medium could be used as an effective transport medium. Mycoplasmas are resistant to penicillin and 500 – 1 000 units per ml were added to the medium to aid in the suppression of bacterial contaminants.

Cultivation of mycoplasmas

Samples were cultivated at the Klein Karoo Research and Diagnostic Laboratory in Oudtshoorn. Swabs were plated onto modified Chanock's agar medium and the plates incubated in candle jars at 37°C for 2 to 21 days. The plates were examined every two days using a stereomicroscope at 10 - 40 x magnification with obliquely transmitted light. Colonies were usually visible 72 hours after inoculation of the plates, although this was found to vary.

Mycoplasma growth was typical of other mycoplasmas, exhibiting the classic fried-egg morphology, although this was not always easily observed, especially in young cultures. The mycoplasmas tended to penetrate the surface of solid media, resulting in a central nucleus that remained embedded in the medium while the rest of the colony (peripheral zone) could be removed by gently scraping over the agar. The agar containing mycoplasma growth was subsequently sent to Stellenbosch for DNA analysis.

Preparation of genomic DNA

DNA was extracted from the mycoplasma-containing solid agar using the N-cetyl-N,N,N-trimethyl ammonium bromide (CTAB) method of Doyle & Doyle (1987). The CTAB method was originally developed for the extraction of genomic DNA from fresh plant tissue. Briefly, 500 µl of a 2 x CTAB buffer (100 mM Tris-HCl, pH 8.0; 1.4 M NaCl; 20 mM EDTA, pH 8.0; 2% v/v CTAB; 0.2% v/v 2-mercaptoethanol) was added to the mycoplasma containing agar and incubated at 60°C for 1 h. To this, 500 µl chloroform-isoamylalcohol (24:1 v/v) was added and gently mixed for 10 min followed by centrifugation for 5 min at 7 000 x g. The upper aqueous phase was removed and

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to this, a 2/3 volume of cold isopropanol was added and mixed gently. This sample was incubated overnight at -20°C to allow for the precipitation of nucleic acids. After incubation, the sample was centrifuged for 2 min at 3 000 x g. The supernatant was decanted and 1.5 ml wash buffer (40 mM ammonium acetate:absolute ethanol; 1:3) added to the pellet. The pellet was resuspended in the wash buffer and incubated at RT for 20 min. This was followed by centrifugation for 1 min at 3 000 x g, after which the supernatant was once again poured off and the pellet air-dried to get rid of the ethanol. The DNA pellet was finally redissolved overnight at 4°C in 500 µl TE-Buffer (10 mM Tris-HCl, pH 8.0; 1 mM EDTA, pH 8.0).

Primer selection for amplification of the 16S rRNA gene

The presence of mycoplasma in each sample was verified using a 16S rRNA based general primer pair (GPO3 and MGSO), used for the identification of mycoplasmas at both genus- and species level (Van Kuppeveld *et al.*, 1992). The GPO3 primer sequence, however, was slightly changed by moving the position of the primer on the 16S rRNA sequence, two basepairs to the left. This was done to avoid the possibility of secondary structure formation of the GPO3 primer (as indicated by the computer program Primer Designer, version 2.0), and was subsequently renamed GPO3F.

The 16S rRNA gene of mycoplasma positive samples was further investigated using 16S rRNA primers developed for *E. coli* (Hauben *et al.*, 1997) in combination with the general mycoplasma primers. The primer combinations of 16F27 and MGSO as well as GPO3F and 16R1541, were used to amplify the entire 16S rRNA gene. All primers were synthesized by the DNA Synthesis Laboratory, Department of Biochemistry, University of Cape Town. Primer sequences are shown in Table 1.

PCR amplification of the mycoplasma 16S rRNA gene

Amplification reactions for primers GPO3F and MGSO were carried out in 20 µl volumes. Each reaction mixture contained 2 µl of 10 x Reaction buffer (RB, Southern Cross Biotechnology), 0.8 µl of 5 mM of each nucleotide (dATP, dCTP, dGTP and dTTP; Southern Cross Biotechnology), 0.4 µl of each primer (20 pmol/µl), 1.6 µl of 25 mM $MgCl_2$, 0.04 µl of Taq DNA Polymerase (0.2 Units; Southern Cross Biotechnology), 12.76 µl Millipore water and 2 µl of the purified DNA.

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Amplification of the 16S rRNA gene using primer pairs 16F27 and MGSO as well as GPO3F and 16R1541 was carried out in 50 µl reaction volumes. Each reaction mixture contained 5 µl 10 x RB, 2 µl of 5 mM of each nucleotide (dATP, dCTP, dGTP and dTTP), 1 µl of each primer (20 pmol/µl), 4 µl of 25 mM $MgCl_2$, 0.1 µl of Taq DNA Polymerase (0.2 Units), 31.90 µl Millipore water and 5 µl of the purified DNA.

All the above DNA amplifications were performed in a Hybaid PCR Express Thermal Cycler. The thermal cycler was programmed to perform 35 cycles of denaturation at 94°C (45 sec), primer annealing at 55°C (45 sec) and primer extension at 72°C (1 min 30 sec). This was followed by a final extension reaction for 6 min at 74°C. PCR products were kept at 4°C until analyzed. A negative control sample was included in each PCR reaction to check for contamination. The negative control was DNA isolated from a mycoplasma culture medium containing agar sample and which gave a negative PCR result.

Table 1: Primers used for amplification and sequencing of ostrich mycoplasmas

PRIMER	SEQUENCE	bp-POSITION	T_m (°C)$
General mycoplasma primers (Van Kuppeveld *et al.*, 1992)			
GPO3F (F)	5'-TGGGGAGCAAACAGGATTAGATACC-3'	745	78
MGSO (R)	5'-TGCACCATCTGTCACTCTGTTAACCTC-3'	1017	79
16S rRNA primers developed for *E. coli* (Hauben *et al.*, 1997)			
16F27 (F)	5'-AGAGTTTGATC(A/C)TGGCTCAG-3'	8*	59
16F530 (F)	5'-T(C/T)(C/T)GTGCCAGCAGCCGCGG-3'	512*	66
16R1541 (R)	5'-GGTTGGATCACCTCCTT-3'	1525*	52

(F) Forward primer.
(R) Reverse primer.
* Numbering of target position based on that of the *E. coli* 16S rRNA sequence.
$ T_m as calculated by the computer program Primer Designer, version 2.0.

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Detection of PCR products

Amplified DNA was analyzed by gel electrophoresis. Ten μl of the PCR product, mixed with a 0.1 volume of gel loading buffer (50% glycerol; 0.1% v/v bromophenol blue; 50 mM EDTA; 100 mM Tris-base, pH 8.0) was separated on a 2% agarose gel (Molecular Grade Agarose D1-LE, Whitehead Scientific) in 1 x TAE buffer (Tris-base; glacial acetic acid; 0.5 M EDTA, pH 8.0). Ethidium bromide (0.175 μg/ml) was included in the gel for ultra-violet (UV) visualization of the DNA.

Sequencing of the mycoplasma 16S rRNA gene

DNA templates for sequencing reactions were prepared by purifying the PCR products of primer pairs 16F27 and MGSO as well as GPO3F and 16R1541. Firstly, 80 μl of each of the PCR products were electrophoresed on a 0.5% agarose gel in 1 x TAE buffer containing ethidium bromide (0.15 μg/ml) as previously described. The DNA containing bands were excised under a UV light and purified using the Promega Wizard PCR Prep Kit. Purification was done according to the manufacturers' instructions except for the final incubation step, which was replaced by two incubation steps of 10 min each at 37°C in order to maximize DNA yield.

The purified DNA samples were each concentrated to 20 μl on a Savant Speedvac and subsequently analyzed by gel electrophoresis. Two μl of the PCR product, mixed with a 0.1 μl volume of gel loading buffer (50% glycerol; 0.1% bromophenol blue; 50 mM EDTA; 100 mM Tris-base, pH 8.0) was separated on a 2% agarose gel in 1 x TAE buffer. Ethidium bromide (0.175 μg/ml) was included in the gel for UV visualization of the DNA.

Sequencing reactions were prepared using the ABI PRISM® BigDye™ Terminator v3.0 Cycle Sequencing Ready Reaction Kit with primers 16F27, MGSO and 16R1541. Amplifications were performed in a Hybaid PCR Express Thermal Cycler, programmed to perform 35 cycles of denaturation at 96°C (10 sec), primer annealing at 52°C (30 sec) and primer extension at 60°C (4min). This was followed by a final extension reaction of 10 min at 60°C. Sequencing PCR reaction products were analyzed with an ABI PRISM® 373 DNA Sequencer at the DNA sequencing facility of the University of Stellenbosch, Stellenbosch, South Africa.

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Phylogenetic Analysis

Sequence data generated using each of the 3 primers were combined to form a single 16S rRNA gene sequence. These sequences were then aligned to and compared with other avian 16S rRNA gene sequences available on the NCBI nucleotide sequence database as well as sequences from other related *Mollicutes* and bacteria. Sequences were initially aligned using the DNA and Protein Sequence Alignment (DAPSA) program (Harley, 1998) and final alignments were made manually.

Phylogenetic analysis of the ostrich isolated mycoplasmas was performed using the programme PAUP* (phylogenetic analysis using parsimony), version 3.10β (Swofford, 1993). *E. coli* was used as an outgroup to root the tree. Support for the phylogenetic tree was evaluated using bootstrap analysis, by resampling the data 1 000 times. From these trees a most parsimonious tree was selected as shown in Figure 3.

Primer development for ostrich mycoplasmas

Based on computer alignments (DAPSA) of the 16S rRNA gene sequences of Ms01, Ms02 and Ms03, primer pairs were selected for each from an area in which the sequence of each mycoplasma was unique (Neefs *et al.*, 1990). In addition to this, the 3'-end of the primers was chosen to further allow maximum specificity and minimize the possibility of cross-reaction between the three ostrich mycoplasmas. Primers were designed using the computer software package, Primer Designer Version 2.0. All primers were synthesized by the DNA Synthesis Laboratory, Department of Biochemistry, University of Cape Town, South Africa. Primer sequences are shown in Table 2.

Optimization of ostrich specific mycoplasma PCR

Following initial trials, optimal cycling times and annealing temperatures for each of the ostrich specific primers were determined in order to yield optimum specificity.

Amplification reactions were carried out in 20 µl volumes. Each reaction mixture contained 2 µl of 10 x RB (Southern Cross Biotechnology), 0.8 µl of 5 mM of each nucleotide (dATP, dCTP, dGTP and dTTP; Southern Cross Biotechnology), 0.4 µl of each primer (20 pmol/µl), 1.6 µl of 25 mM $MgCl_2$, 0.04 µl of Taq DNA Polymerase

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(0.2 Units; Southern Cross Biotechnology), 12.76 µl Millipore water and 2 µl of isolated DNA.

DNA amplifications were performed in a Hybaid PCR Express Thermal Cycler. The thermal cycler was programmed to perform 30 cycles of denaturation at 94°C (30 sec), primer annealing at 57°C (15 sec) and primer extension at 72°C (1 min). This was followed by a final extension reaction for 6 min at 74°C. PCR products were kept at 4°C until analyzed. PCR products were separated by gel electrophoresis as previously described.

Table 2: Ostrich mycoplasma specific primers

PRIMER	SEQUENCE	bp-POSITION	T_m (°C)$
Ms01			
MS012 (F)	5'-AACATTAGTTAATGCCGGATACGC-3'	114	75
MS01D (R)	5'-GCCAGTATCCAAAGCGAGCC-3'	613	75
Ms02			
MS023 (F)	5'-GTAATGGCTCACCAAGACGA-3'	227	69
MS02B (F)	5'-CTAATGCCGAATACTTATTAGC-3'	122	65
MS02H (F)	5'-AATATAAAAGGAGCGTTTGC-3'	160	70
MS02A (R)	5'-AAGGCAATAGCATTTCCTCTACT-3'	447	70
Ms03			
MS035 (F)	5'-AGCGACACAGCGTGCAGGAA-3'	360	78
MS03D (F)	5'-GGCAAGGCCTTGACAGTACTAA-3'	437	72
MS03F (F)	5'-GATGATTAGCTGATAGGAACTATCG-3'	792	70
MS03A (F)	5'-AGTGCTAATGCCGGATACTTATAC-3'	118	70
MS03C (R)	5'-CGTTAACCTCTATACAATTCTAGCG-3'	639	70

(F) Forward primer.
(R) Reverse primer.
$ T_m as calculated by the computer program Primer Designer, version 2.0.

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RESULTS

Genomic DNA isolation

Mycoplasma DNA could be isolated successfully from the solid agar medium using the CTAB method. In instances where broth cultures were received they were used directly in the PCR reaction without prior DNA isolation.

Amplification of the 16S rRNA gene

Mycoplasma containing samples were identified using a general mycoplasma primer pair followed by amplification of the entire gene as illustrated in Figure 1. PCR amplification using the general mycoplasma primers, produced a product of 270 bp as expected. Amplification of the entire gene using the primer combinations 16F27 and MGSO as well as GPO3F and 16R1541 produced amplification products of 1048 and 769 bp respectively. Positive amplification products using the general mycoplasma primers were only obtained for samples isolated from the upper respiratory system, whilst none of the samples isolated from either the jejunum, ileum or colon gave a positive PCR product.

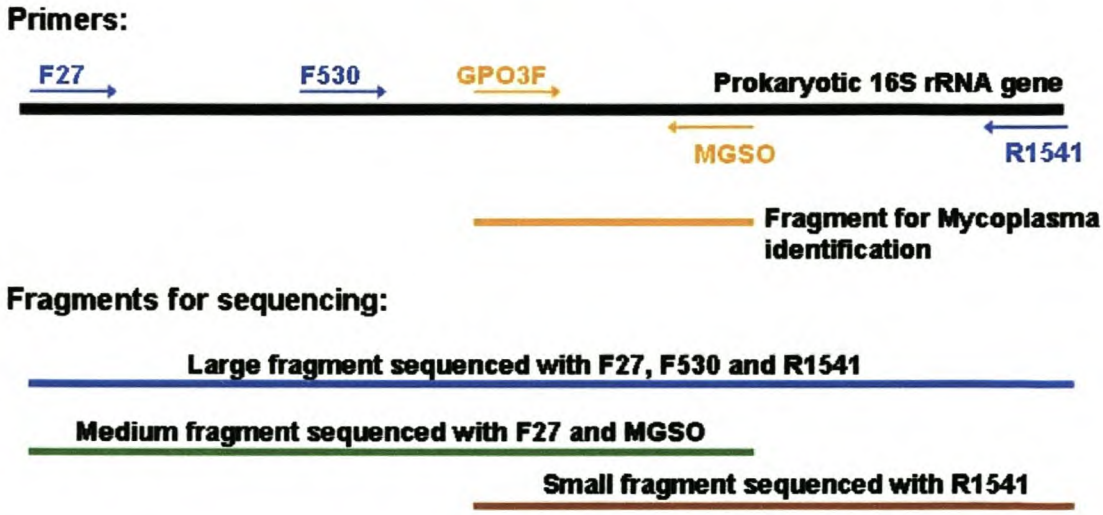

Figure 1: Strategy used for the amplification and sequencing of the mycoplasma 16S rRNA gene. The 16F27, MGSO and 16R1541 primers were subsequently used for the amplification of the entire mycoplasma 16S rRNA gene.

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Sequencing of mycoplasma 16S rRNA genes from ostrich and chicken samples

Three ostrich mycoplasmas were identified from the 242 samples analyzed by specific sequencing of the 16S rRNA gene. Mycoplasmas are usually named according to their host or disease pathology and in this study ostrich mycoplasmas were provisionally named '*Mycoplasma struthiolus*' (Ms) after their host, *Struthio camelus*. Thus, the three mycoplasmas were designated Ms01, Ms02 and Ms03 respectively until formally described. Requirements for the formal description of a new *Mollicutes* species include a variety of biochemical tests as laid down by the *Mollicutes* Taxonomy Committee (Whitcomb *et al.*, 1995). Before these tests can be conducted, the relevant mycoplasmas have to be cultivated and cloned. Due to the difficulties experienced in cultivation of all three ostrich mycoplasmas, these tests have not yet been performed. From the 242 samples analyzed, 52 were Ms01, 26 Ms02 and 137 Ms03. Fifteen of the samples had mixed infections of Ms01 and Ms03 and 4 of Ms02 and Ms03. Except for one ostrich sample containing *M. gallisepticum*, no other known poultry or avian mycoplasmas were isolated from any of the ostrich samples. None of the chicken DNA samples that were isolated and sequenced contained any of the three ostrich mycoplasmas, but instead contained *M. gallinaceum* and *M. pullorum*. The different districts from where the ostrich mycoplasmas were obtained are shown in Figure 2. The different ostrich mycoplasmas isolated in each of these districts are shown in Table 3.

Phylogenetic analysis

Alignment data showed 88.4% sequence similarity between Ms01 and Ms02, 88.7% sequence similarity between Ms01 and Ms03 and 93.1% sequence similarity between Ms02 and Ms03 respectively.

Parsimony analysis produced 14 trees of a length of 2173 steps, with a consistency index of 0.543 and a retention index of 0.684. From these trees a representative most parsimonious tree was chosen as shown in Figure 3. Nodal support was assessed by bootstrap analysis and the bootstrap percentages, as calculated from the 1 000 replicates, are given at the individual nodes of the tree. Only bootstrap values above 70% were regarded as significant.

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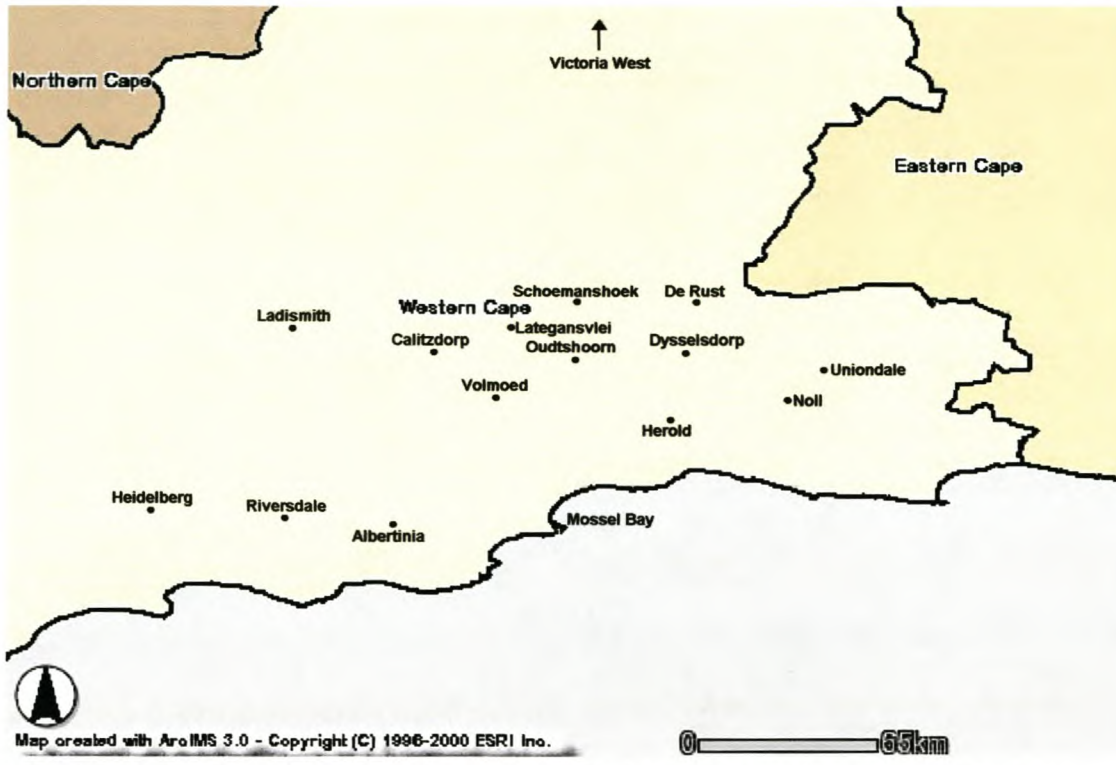

Figure 2: A map showing the different districts from where ostrich mycoplasma samples were obtained in the Western Cape and Northern Cape Province.

Table 3: Incidence of Ms01, Ms02 and Ms03 in each of the districts from where samples were obtained

DISTRICT	MYCOPLASMA				
	Ms01	Ms02	Ms03	Ms01 & Ms03	Ms02 & Ms03
Klein Karoo					
Ladismith	7/32	2/32	18/32	5/32	-
Calitzdorp	15/27	2/27	8/27	2/27	-
Oudtshoorn	14/77	13/77	47/77	-	3/77
Volmoed	2/30	7/30	20/30	-	1/30
Dysselsdorp	-	-	-	-	-
De Rust	8/20	-	9/20	3/20	-
Uniondale	-	-	2/2	-	-
Noll	-	-	1/1	-	-
Schoemanshoek	-	-	7/7	-	-
Herold	-	-	2/2	-	-
Lategansvlei	1/12	-	11/12	-	-
Garden Route					
Heidelberg	-	½	½	-	-
Riversdale	-	-	2/2	-	-
Albertinia	-	-	1/1	-	-
Mossel Bay	5/17	1/17	6/17	5/17	-
Central Karoo					
Victoria-West	-	-	2/2	-	-

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Phylogenetic analysis showed Ms02 and Ms03 to fall together in one clade with Ms02 closely related to *M. gallinaceum* and Ms03 closely related to *M. synoviae*. Ms01 falls into a separate clade with its closest relative being *M. falconis*. According to the alignment data, Ms02 and *M. gallinaceum* have 95.2%, Ms03 and *M. synoviae* 92.6% and Ms01 and *M. falconis* 97.7% sequence similarity respectively.

Primer development for ostrich mycoplasmas

Alignment of the 16S rRNA sequences of Ms01, Ms02 and Ms03 allowed the identification of regions with conserved and variable sequences. Potential primers specific for each of the ostrich mycoplasmas were selected from these variable areas. Despite the fact that the last three bases of the 3'-end of each primer were chosen to minimize non-specific binding of primers, i.e. to be unique for each ostrich mycoplasma, some of the primers were still able to bind to a different mycoplasma sequence. Primer MS023 gave a positive PCR product with Ms03 samples and MS02B with Ms03 and Ms01 samples. Primers MS035 and MS03D gave positive PCR products with Ms02 samples and MS03F gave positive PCR products with Ms02 and Ms01 samples. The binding specificity of these primers could not be improved by changing any of the PCR conditions. Primer pairs, which gave the necessary specificity for Ms01, were MS012 and MS01D, for Ms02, MS02H and MS02A and for Ms03, primers MS03A and MS03C, respectively (Table 2).

Ostrich mycoplasma specific PCR assay

The specific primer pairs for Ms01, Ms02 and Ms03 produced PCR products of 494, 288 and 517 bp respectively as seen on agarose gel electrophoresis (Figure 4). Each of the 242 ostrich samples was tested using the ostrich specific mycoplasma primers. The specific PCRs were able to successfully differentiate between the three different ostrich mycoplasmas as expected from previous sequencing results. Where sequencing was only able to identify one mycoplasma in samples containing mixed infections, the specific primers were able to identify these mixed infections. Only one sample was identified as having a mixed infection on the basis of sequencing results only.

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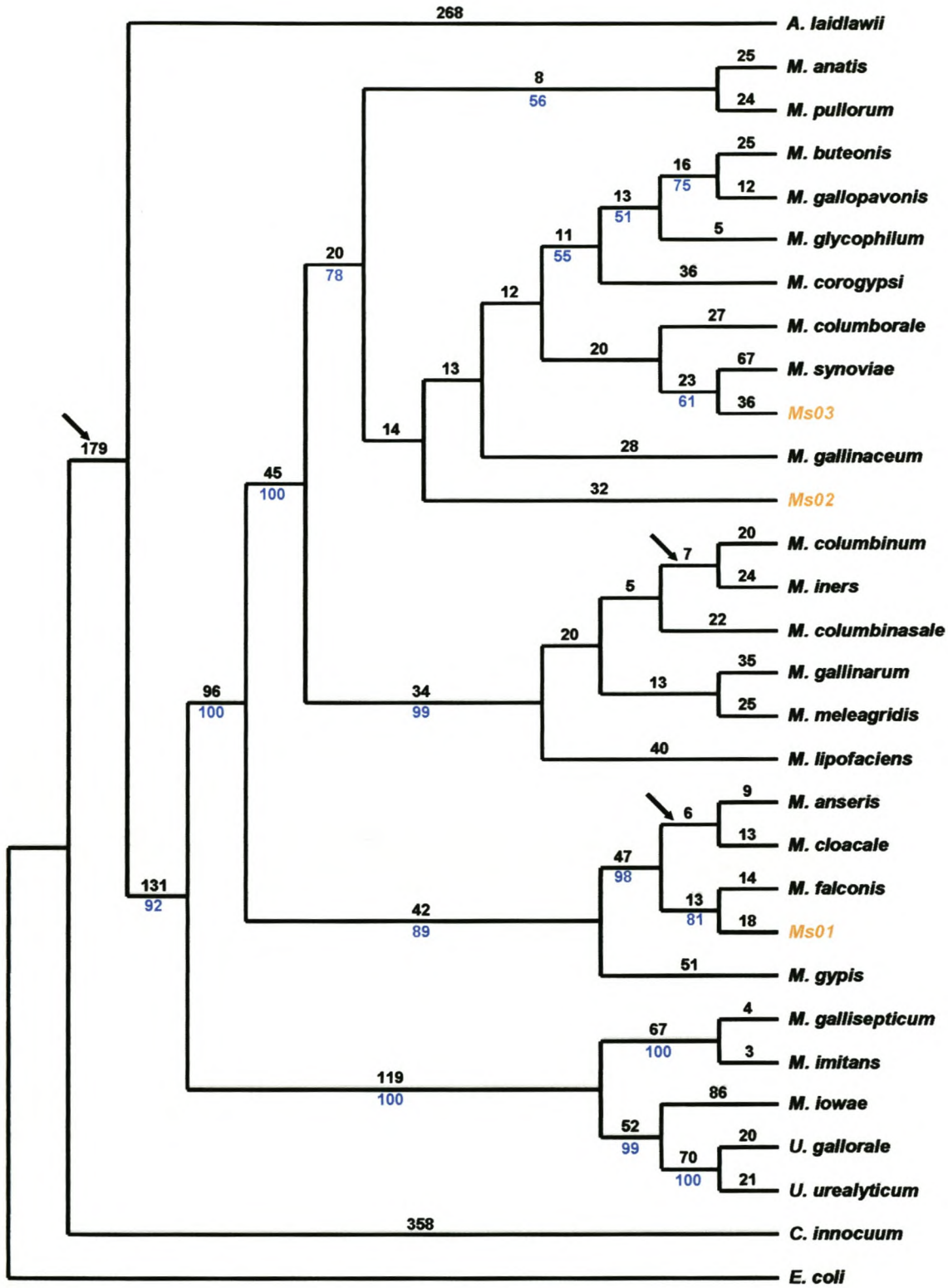

Figure 3: A selected most parsimonius 16S rRNA gene tree of avian mycoplasmas. Branch lengths and Bootstrap values are indicated above and below the line respectively. Arrows indicate branches that collapse in the strict consensus tree. Bootstrap values below 50 are not shown.

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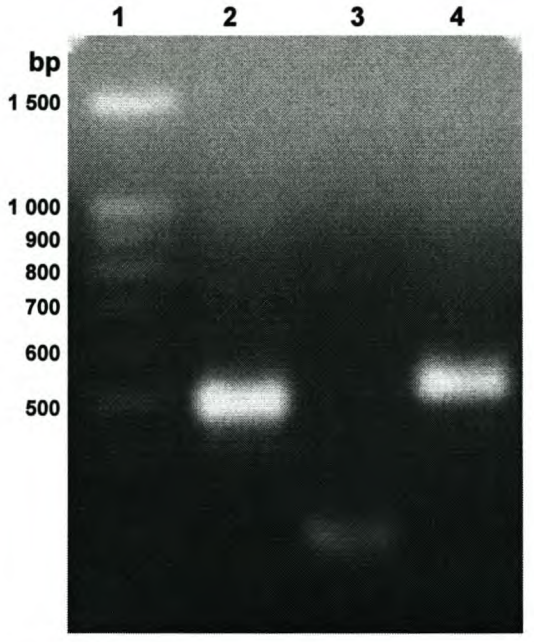

Figure 4: 2% Agarose gel containing PCR products from samples containing ostrich specific mycoplasmas. These products were produced using Ms01 (lane 2), Ms02 (lane 3) and Ms03 (lane 4) specific primers. Lane 1 contains a 100 bp DNA ladder (Promega).

DISCUSSION

The identification of mycoplasmas by 16S rRNA gene sequencing showed that ostriches carry unique mycoplasmas and are apparently not infected with domestic poultry mycoplasmas. Three mycoplasmas were isolated from ostriches and provisionally named Ms01, Ms02 and Ms03 until formally described. The single ostrich from which *M. gallisepticum* was isolated, was from an ostrich farm where the ostriches were kept in close proximity to chickens. This is in agreement with Peccati *et al.* (1996) who found that ostriches, kept in paddocks together with backyard poultry, became infected with poultry mycoplasmas. As a result of the susceptibility of ostriches to poultry mycoplasmas it is recommended that ostrich farms be kept free of any poultry (Verwoerd, 2000). None of the chickens that were tested were infected with any of the three ostrich mycoplamas, but were infected with *M. gallinaceum* and *M. pullorum*. Ms03 was found to be widely distributed in all of the districts sampled in the Central Karoo, Klein Karoo and Garden Route area of the Northern and Western Cape Province. Although Ms01 and Ms02 were found in fewer districts, no specific geographic trends could be established. The most likely reason for this is because

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ostriches are often moved from one farm to another, which would disturb any specific natural distribution pattern. Where two types of mycoplasmas occurred together, mixed infections were also often found.

The fact that ostrich mycoplasma DNA could successfully be isolated and amplified from samples of the upper respiratory system and eyes compared to the alimentary tract, illustrates the respiratory nature of mycoplasma infections in ostriches. The highest incidence of Ms01 was found in samples collected during the winter and springs months (June – December), whilst the highest incidence of Ms02 and Ms03 was during June and July and again in November. Although mycoplasma infections occur right throughout the year the highest incidence appears to be during the cold winter months and again at the start of summer. Apparently, extreme environmental temperatures or sudden changes in environmental temperatures (Autumn to Winter or Spring to Summer) can stress the birds thereby leaving them vulnerable to subsequent infections. In poultry, the incidence of mycoplasma infections is also influenced by environmental conditions and infections are also known to increase during the winter months (Jordan, 1996; Kleven, 1998).

The 16S rRNA gene was found to be a convenient genetic marker to compare the phylogenetic relationships among avian mycoplasmas. Phylogenetic analysis of the 16S rRNA gene sequences of the three isolated ostrich mycoplasmas, showed them to be quite divergent and fall into two distinct phylogenetic mycoplasma groupings. Phylogenetic analysis indicated Ms03 to be closely related to *M. synoviae*. This may explain why previous identifications using the FAT indicated the presence of *M. synoviae* in ostriches. Ms02 appeared in the same clade as Ms03, but was still clearly distinct from its closest relative *M. gallinaceum*, a typical poultry mycoplasma. Ms01, however, appears to be phylogenetically distinct and falls in a completely different clade with *M. falconis* being its closest relative.

Differences between the 16S rRNA sequences of the ostrich mycoplasmas were used for the development of ostrich mycoplasma specific primers. The ostrich mycoplasma specific primers could be used to identify mixed infections in samples that were not identified by sequencing. The reason for this may be that some genes are preferentially amplified during PCR and the frequency of a specific sequence, prepared from environmental samples, therefore does not reflect its relative abundance in the microbial community (Wang and Wang, 1996).

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PCR is an ideal method for the diagnosis of diseases where the infectious agents, such as mycoplasmas, are time-consuming to isolate and difficult to identify (Kiss *et al.*, 1997). It has the further advantage of being able to detect infections before symptoms are visible (Moalic *et al.*, 1997). For this reason, the ostrich mycoplasma specific PCR assays will be valuable in determining the prevalence and epidemiology of mycoplasma infections thereby assisting disease management in ostriches. Currently, the specific PCR assays developed in this study are being used in the research laboratory of the Klein Karoo Co-operative in Oudtshoorn to routinely identify all mycoplasma infections.

Control of mycoplasma infections in poultry has been brought about by effective vaccination. The knowledge of the phylogenetic relationships of the three ostrich mycoplasmas to other avian mycoplasmas can be used in establishing vaccination strategies for the control of mycoplasma infections in ostriches. Thus we plan to investigate the efficacy of *M. synoviae* vaccines to give protection against Ms03 and possibly Ms02. Vaccination strategies using *M. gallisepticum* vaccines have been unsuccessful, which is borne out by the phylogenetic analysis which shows that *M. gallisepticum* falls in a group of mycoplasmas only distantly related to the three ostrich mycoplasmas. However, as no poultry mycoplasma vaccines are available for close relatives of Ms01, a specific vaccine will have to be developed.

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CHAPTER 6:

Avian malaria in the African penguin

6.1. THE AFRICAN PENGUIN

The African penguin (*Spheniscus demersus*), commonly known as the Jackass penguin (named for its donkey-like braying call), Cape penguin or Black-footed penguin, is the only penguin species to breed in Africa (Payne and Crawford, 1992; Hockey, 2001). Its nearest relatives, the Humboldt penguin (*S. humboldti*) and Magellanic penguin (*S. magellanicus*), are almost 9 000 km away along the coast of southern South America. The only other close relative is the Galapagos penguin (*S. mendiculus*), the world's most tropical penguin and found on the Galapagos Islands (Hockey, 2001). Although confined largely to the cold waters of the extreme southwest, the African penguin has a breeding range from as far north and west as Hollams Bird Island in Namibia (24°37'S 14°31'E) to Bird Island at Algoa Bay in the Eastern Cape (33°50'S 25°50'E). African penguins do, however, regularly move north and east of their breeding range, especially to coasts of northern Namibia and Southern Angola, but have also been seen as far north as Gabon and Southern Mozambique (Randall, 1989; Payne and Crawford, 1992; Hockey 2001).

Besides the availability of nutrient-rich waters as provided by the cool, northward-flowing Benguela current, the distribution of the African penguin is determined by the availability of offshore islands for breeding sites and currently there are 24 island and 3 mainland colonies. At the start of the 20th century, it is likely that there were more than 1.45 million African penguin adult birds. By the early 1990s, this number had dropped to 179 000 adults, and at present the population is about 10% of that at the start of the 20th century (Nel *et al.*, 2003). Despite the overall pattern of decline, there are some colonies where the numbers of penguins are increasing. There is, however, still some concern for colonies on islands in Southern Namibia and Dyer Island, west of Cape Agulhas (Hockey, 2001). In a period of 25 years, starting in the late 1970s, there has been a total decrease in breeding pairs of more than 85% on these islands. This decline in penguin populations is due to threats from both human and natural sources (Hockey, 2001).

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6.1.1. HUMAN THREATS

Historically human threats started as early as the 15th century when Africa's penguins became the first of the world's penguins to be discovered by Europeans (Randall, 1989; Hockey, 2001). In the following centuries these birds provided a ready source of meat and eggs for visiting seafarers (Randall, 1989; Hockey, 2001).

In the early 19th century the South American guano rush spread to Africa. These guano scrapings continued on some of South Africa's islands right up until 1991. The initial harvests had a major impact on the penguin populations, with repercussions that have lasted well into the present (Randall, 1989; Hockey, 2001).

At the beginning of the 20th century the penguin populations were once again under human threat. There was a wholesale commercial collection of penguin eggs for food, initially as a cheap source of protein for the poor and with increasing scarcity they became a luxury food. Even before this practice ceased in 1968, the African penguin was faced with a depletion of their food supplies due to the west-coast 'fish boom' of the post-war years. This caused a crash in the African penguin populations between Table Bay and Lüderitz (southern Namibia) (Randall, 1989; Payne and Crawford, 1992; Hockey, 2001).

Modern day human threats include commercial fishing and oil pollution (Randall, 1989; Payne and Crawford, 1992). Although the first oiled penguin was sighted as early as the 1920s (Kearton, 1930), oiling only became a serious threat after the closing of the Suez Canal to tanker traffic due to Arab-Israeli conflicts. This resulted in a re-routing of oil tankers, travelling from the Arabian Gulf to Europe, around Africa. Trying to save costs on crewing the tankers, small tankers were soon replaced with super-tankers. In an attempt to reduce transport costs and to pick up fresh supplies, these vessels sail very close to the South African coast. Therefore, despite a reduction in the number of tankers moving around the African coast, the size of these tankers along with their unsuitability for the rougher fully oceanic conditions of the South African coast, subsequently increases the potential magnitude of oil spills, should these tankers become damaged (Williams, 2003). Between 1985 and 2000 there were four major oil spills around the South African coast. The last of these was the bulk ore carrier, Treasure, which sank off Robben Island on 23 June 2000, causing the largest seabird rescue mission the world has ever seen. Approximately 20 000 adult and immature penguins were oiled in the process, representing about

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12% of the global population (Hockey, 2001; Nel *et al.*, 2003). African penguins are particularly susceptible to marine oil pollution due to them being flightless and spending most of their time in the sea, at or near the ocean surface. Oiling causes the penguins' feathers to clump, breaking down their natural insulative and waterproofing properties. Loosing their insulation, these birds will come ashore and not return to sea to forage. If not rescued, these penguins will eventually die from hypothermia and dehydration or even starvation. In an attempt to remove the oil by preening themselves, they can ingest oil that in turn can lead to ulceration of internal organs, anaemia, petro-chemical poisoning and altering of immune functions (Briggs *et al.*, 1996; Nel *et al.*, 2003).

6.1.2. NATURAL THREATS

Natural threats to penguins include: sharks, Killer Whales, Cape Fur Seals, Large-spotted Genets, leopards, Kelp Gulls, Sacred Ibises, mole snakes and even domestic cats (Randall, 1989; Payne and Crawford, 1992; Hockey, 2001). Breeding in colonies does have certain advantages such as providing protection against predators, but unfortunately it also has disadvantages such as easy transmission of diseases and parasites. Generally diseases seem to be more of a problem for captive penguins than for those in the wild. Diseases known to affect and kill penguins include: avian cholera, babesia, Newcastle disease and avian malaria (Alexander *et al.*, 1989; Brossy, 1992; Hockey, 2001).

6.1.3. REHABILITATION OF PENGUINS

The African penguin population is about 10% of that at the beginning of the 20th century (Nel *et al.*, 2003). A decrease in the population size of the African penguin as well as being concentrated within relatively small geographic areas causes them to become more vulnerable to any event that can trigger further decline (Stoskopf, 1993). Therefore, events such as oil spills, affecting thousands of birds, are seen as an important immediate threat to African penguin populations (Nel *et al.*, 2003). The South African Foundation for the Conservation of Coastal Birds (SANCCOB), established in 1968, is a seabird rescue and rehabilitation centre located at Tableview in Cape Town. Although dedicated largely to the de-oiling of penguins, both penguins and other sea birds, which are found along the coast either diseased or injured, are also brought to this facility for treatment. These birds are then released as soon as they are able to fend for themselves (Brossy, 1992). A large percentage

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of penguins that have been rehabilitated and released not only survive well, but they return to their own colonies, former nests and mates and continue to breed successfully (Randall *et al.*, 1980; Brossy, 1992; Hockey, 2001; Whittington, 2003; Wolfaardt and Nel, 2003). SANCCOB has played a vital role in the rehabilitation of oiled penguins. In fact, demographic impact studies showed that, largely due to the de-oiling efforts of SANCCOB, the 2002 population of African penguins is now 19% larger than it would have been without rehabilitation (Ryan, 2003).

Despite the success of SANCCOB in rehabilitating penguins, they have significant mortalities. Looking into the cause of death, a high prevalence of avian malaria was found (Brossy, 1992), but how and where these penguins become infected is not certain. More recently, a SANCCOB penguin report by Parsons (2001) stated that approximately 27% of deaths of admitted penguins at SANCCOB can be attributed to avian malaria.

6.2. AVIAN MALARIA AND THE CONSERVATION OF THE AFRICAN PENGUIN

The potential impact that avian malaria can have on wild avian populations is clearly illustrated by the history of the indigenous birds of the Hawaiian Islands. In 1826 mosquitoes (*Culex quinquefasciatus*) were introduced to the region by a sailing vessel stopping at Lahaina, Mauaia for refilling of water kegs (Garnham, 1966; Van Riper *et al.*, 1986; Anon, 2002). Although the mosquitoes rapidly invaded the islands, migratory birds were not a substantial reservoir of avian malaria at that stage and avian malaria only reached epizootic proportions early in the 1900's following the introduction of foreign bird species (Van Riper *et al.*, 1986). Subsequent transmission of avian malaria from infected avian hosts resulted in the extinction of many indigenous Hawaiian bird species. Currently diseases such as avian malaria, along with fowl poxvirus, have been reported as factors responsible for the decline of native Hawaiian bird populations as well as influencing avian population patterns on the Islands (Garnham, 1966; Van Riper *et al.*, 1986; Massey *et al.*, 1996). One of these birds is the Hawaiian crow or Alalã (*Corvus hawaiiensis*), which is indigenous to the Hawaiian Islands and a severely endangered species (Massey *et al.*, 1996). In 1995, the wild population was estimated to consist of less than 20 birds with only 3

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reproductively active pairs. The captive birds numbered 14 of which only 2 pairs produced eggs (Massey *et al.*, 1996).

Currently the status of the African penguin is listed as "Vulnerable" in both the South African Red Data Book for birds (Barnes, 2000) and the IUCN Red List of Threatened Species™ (http://www.redlist.org). It is also listed in Appendix II of both the Convention on Trade in Endangered Species (CITES) and the Convention on the Conservation of Migratory Species of Wild Animals (the Bonn Convention) (Underhill, 1996). Notwithstanding the success of SANCCOB in the rehabilitation and thus conservation of African penguins, the possibility of avian malaria being contracted by penguins at the rehabilitation facility causes concern as to the dangers of introducing avian malaria into already endangered wild populations (Brossy, 1992; Graczyk *et al.*, 1995a; Hockey, 2001). Knowledge concerning the impact of avian malaria on African penguin populations is therefore needed for both effective management of this disease during rehabilitation as well as improving future conservation efforts.

6.3. AVIAN MALARIA AND THE AFRICAN PENGUIN

Avian malaria is a common mosquito transmitted disease in wild and domesticated birds, caused by a protozoan referred to as *Plasmodium* (Atkinson and Van Riper, 1991; Atkinson, 2001a). Malaria parasites in wild birds were first discovered in 1884, at Kharkov in the Ukraine by a Russian scientist named Danilewsky (Garnham, 1966). Since then, avian malaria has been of interest to parasitologists and malariologists as models for studying various aspects of human malaria such as vertebrate-parasite interactions and more importantly, the screening of human anti-malaria chemotherapy agents (Huff, 1963; Atkinson and Van Riper; 1991). The discovery of *Plasmodium* species that infect rats and mice, has, however, replaced the experimental use of avian hosts with inexpensive, easily obtainable and more practical mammalian hosts for malaria research (Seed and Manwell, 1977; Phillips, 1983).

Although the number of *Plasmodium* species infecting birds is far less than that of mammals, avian malaria has a much wider host range and is found across all continents. This fact is the consequence of the vast migratory flights of birds resulting in exposure of these birds to a variety of mosquito species. Parasites are

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subsequently carried across oceans and deserts and provided that a suitable vector is present, avian malaria is then spread amongst non-migratory birds (Garnham, 1966; Seed and Manwell, 1977).

Avian malaria in the African penguin is caused mainly by *P. relictum* and *P. elongatum* parasites, with *P. relictum* being the more virulent strain (Fantham and Porter, 1944; Fleischman *et al.*, 1968; Cranfield *et al.*, 1990; Brossy, 1992; Graczyk *et al.*, 1994d). Single reports of other *Plasmodium* species infecting African penguins include *P. cathemerium* (Bennett *et al.*, 1993) and *P. juxtanucleare* (Grim *et al.*, 2003). The similarity of *P. cathemerium* to other members of the *relictum* group has undoubtedly led to mistaken identification in the past and recent years (Garnham, 1966; Laird, 1998). The fact that *P. cathemerium* closely resembles *P. relictum*, casts some doubt on whether or not this single report of a *P. cathemerium* infection in an African penguin is in fact valid. The following sections contain an overview on different aspects of the epidemiology of avian malaria with specific reference to the African penguin. Discussions will, however, be restricted to *Plasmodium* species infecting African penguins.

6.3.1. CLASSIFICATION AND CHARACTERISTICS

Plasmodia are single-celled intracellular parasitic protozoans and despite the fact that they consist of a single cell, they are amazingly complex in structure, physiology and behaviour (Noble and Noble, 1982). *Plasmodium* parasites can be classified as follows (Garnham, 1966; Vivier and Desportes, 1990; Atkinson and Van Riper, 1991; Margulis and Schwartz, 1998; Atkinson, 2001b):

Superkingdom: Eukaryotae

Kindom: Protoctista (Greek *protos*, very first; *ktistos*, to establish)

Phylum: *Apicomplexa* (Latin *apex*, summit; *complexus*, an embrace, enfolding)

The Apicomplea are subdivided into three classes: Gregarinia, Coccidia and Hematozoa. Members of this phylum are symbiotrophs and the invasive stages (i.e. sporozoites and merozoites) contain a so-called "apical complex" which is important for host cell penetration. The "apical complex" consists of a distinctive arrangement of fibrils, microtubules, vacuoles and other cell organelles at one end of each cell that penetrate tissue in order to obtain nutrients from the host animal.

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Class: *Hematozoa*

According to the classification of Levine *et al.* (1980), *Plasmodium* species belong to the class Coccidia but, although the gamogony of the Hematozoans is of the coccidian type (female gamont gives rise to single gamete), they are considered by Vivier and Desportes (1990) to be a distinct order owing to peculiarities of their sporogony and cytological features.

Order: *Haemosporidia*

Parasites belonging to this order have two hosts, a vertebrate and invertebrate host (Seed and Manwell, 1977).

Family: *Plasmodiidae*

Parasites belonging to this family have a sexual phase in the mosquito and asexual cycles in the tissue and blood cells of the vertebrate host. Gametocytes are produced and confined to mature erythrocytes of the vertebrate host. Parasites within the erythrocytes metabolize haemoglobin and in the process produce the pigment haematin as by-product (Garnham, 1966).

Genus: *Plasmodium*

The *Plasmodium* genus is further divided into subgenera using criteria that relate firstly to the host, then the size of the erythrocytic schizont, the shape of the gametocyte, and finally to the site and growth rate of exoerythrocytic schizonts together with the number of merozoites produced. The genus *Plasmodium* can accordingly be divided into nine subgenera of which four occur in birds, three in mammals and two in lizards. The four subgenera of avian *Plasmodium* are as follows (Garnham, 1966; Seed and Manwell, 1977; Bennett *et al.*, 1993):

Hemamoeba: Type species *relictum*, with large erythrocytic schizonts and round gametocytes (or nearly so) and mature parasites typically displace host cell nucleus towards the cell pole. *P. cathemerium* is a member of this subgenus.

Huffia: Type species *elongatum*, with large erythrocytic schizonts, gametocytes are elongated, mature parasites do not displace the host cell nucleus towards pole and members of this subgenus have a profound attraction to the immature haemopoietic system.

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Giovannolaia: Type species *circumflexum*, with erythrocytic schizonts usually larger than erythrocyte nucleus and contains noticeable amounts of cytoplasm and gametocytes are elongated.

Novyella: Type species *vaughani*, with erythrocytic schizonts smaller than the nucleus and without noticeable cytoplasm and with elongate or oval gametocytes. *P. juxtanucleare* is a member of this subgenus.

6.3.2. EPIDEMIOLOGY

6.3.2.1. Parasite Life Cycle

The *Plasmodium* parasite has a complex life cycle based on alternating cycles of sexual (gametogony) and asexual (schizogony and sporogony) reproduction involving a vertebrate (avian) and invertebrate (mosquito) host. The following outline of the *Plasmodium* life cycle is based on that of *P. relictum*. Given the availability of information and where relevant, specific reference will be made to *P. elongatum*, *P. juxtanucleare* and *P. cathemerium* (Garnham, 1966; Seed and Manwell, 1977; Atkinson and Van Riper, 1991; Atkinson, 2001b; Atkinson, 2001a). A schematic representation of the *Plasmodium* life cycle is shown in Figure 6.1.

A. Stages in the avian host:

The life cycle in the host begins when the infective stages of the parasite (sporozoites) are inoculated into the susceptible host along with salivary secretions of the bloodsucking mosquito. The sporozoites invade macrophages and fibroblasts near the site of the mosquito bite and the now called cryptozoites undergo the first generation of asexual schizogony. Maturation of schizonts (also referred to as segmenters once schizogony is complete) is reached in about 36 – 48 hours with subsequent releasing of merozoites.

The merozoites invade cells of the lymphoid macrophage system as well as the fibroblasts in the brain, spleen, kidney, lung and liver tissue. The order of tissue preference differs between species, e.g. the tissue preference of *P. elongatum* was shown to be the lungs, spleen, liver and heart with fewer schizonts found in kidneys, brain, intestine, bone marrow and skeletal muscle. These were results from post-mortem histological features of penguins that died of *P. elongatum* in the Baltimore Zoo (Fleischman *et al.*, 1968). The now referred to as metacryptozoites undergo a

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second generation of asexual schizogony. Exoerythrocytic schizonts have been reported for *P. juxtanucleare* in the lymphoid-macrophage cells of the spleen, liver, kidney, heart, lung, bone marrow, testes, pancreas and brain, but is most common in the spleen. These first two generations of schizogony are referred to as primary exoerythrocytic schizogony. The time taken for the primary exoerythrocytic cycle in *P. relictum* is slightly more rapid compared to other avian species. Fix *et al.* (1988) have shown the *P. relictum* life cycle in Magellanic penguins to have a rapid onset and unusual behaviour that may influence the temporal variability of different life cycle stages.

The merozoites that are released from the metacryptozoites are capable of invading either the circulating erythrocytes or the capillary endothelial cells of the major organs. Merozoites that continue with a third generation of schizogony in fixed tissues of the host (secondary exoerythrocytic schizogony) are referred to as phanerozoites. Merozoites that are released from the phanerozoites can either invade circulating erythrocytes or reinvade endothelial cells of fixed tissues and subsequently undergo additional generations of schizogony. *P. elongatum* does not undergo exoerythrocytic schizogony in capillary endothelial cells of major organs, but instead chooses the haematopoietic tissues of the host. The choice of which organ tissues will be invaded by phanerozoites depends on the strain of parasite as well as the avian host.

Those merozoites from both primary and secondary schizogony that invade the erythrocytes develop into trophozoites, which can either through the process of schizogony produce mature schizonts containing merozoites or through the process of gametogony develop into male (micro-) and female (macro-) gametocytes that are infective to the mosquito vector. In *P. relictum the* erythrocytic cycle is completed in approximately 30 – 36 hours, producing 8 – 32 merozoites or gametocytes depending on the *Plasmodium* strain and species. The erythrocytic cycle for *P. cathemerium*, *P. elongatum* and *P. juxtanucleare* is approximately 24 hours. However, *P. cathemerium* gametocytes require an additional 6 – 8 hours to reach maturity.

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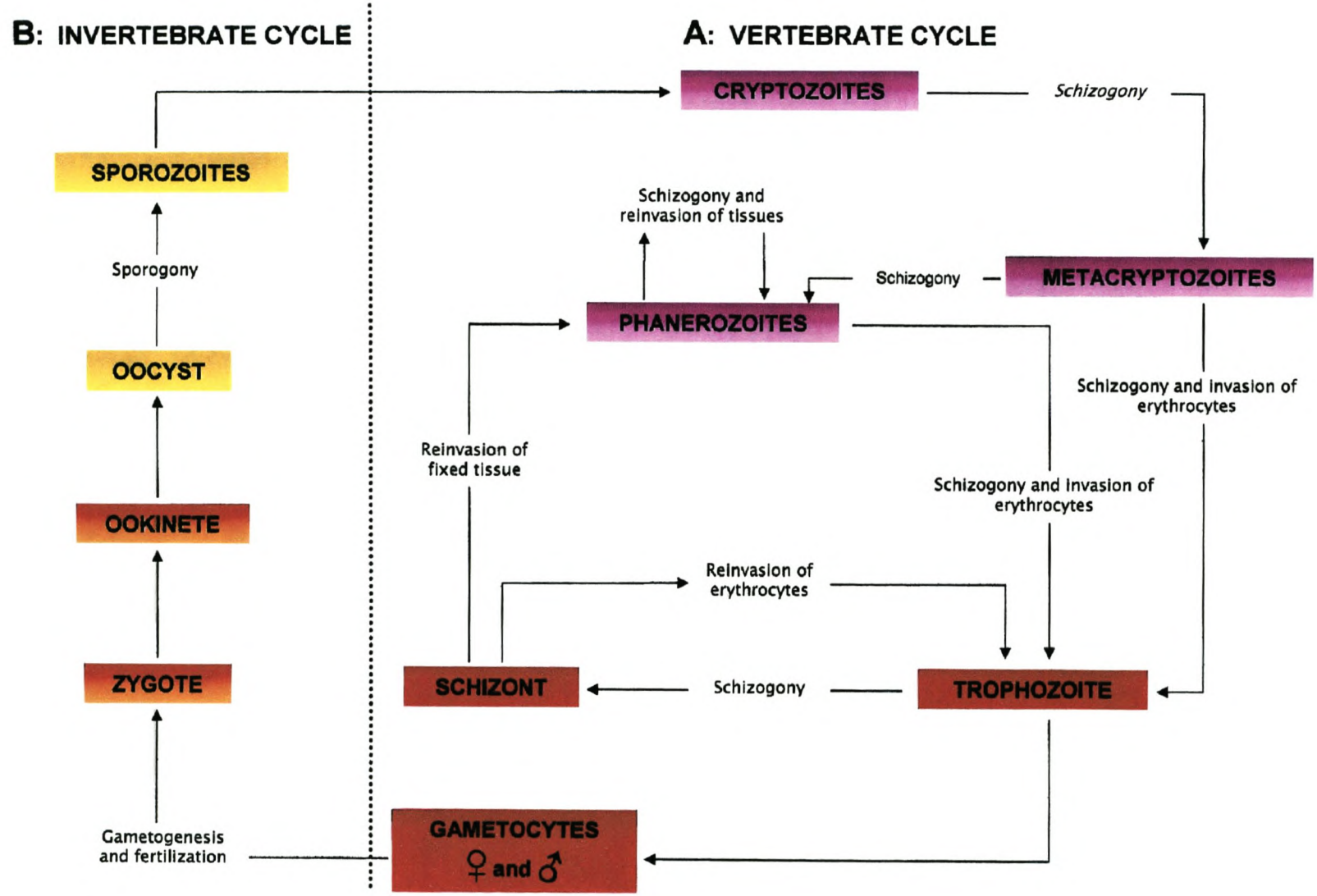

Figure 6.1: *Plasmodium* life cycle in the avian **(A)** and vertebrate host **(B)**.
Adapted from Atkinson (2001b)

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The merozoites that are released from the erythrocytes can either reinvade other erythrocytes and continue schizogony indefinitely in the peripheral circulation or reinvade fixed tissues and thus continue development as phanerozoites. The gametocytes on the other hand remain in the erythrocytes until a suitable vector ingests the infected blood. After erythrocyte invasion by *P. elongatum* exoerythrocytic schizonts are found primarily in the haemocytoblasts and other erythrocyte precursors in bone marrow. *P. elongatum* merozoites from both the erythrocytic and exoerythrocytic schizonts have been found to preferentially invade reticulocytes. Even the gametocytes of *P. elongatum* were found to develop in both erythrocytes and normoblasts (Huff and Bloom, 1935). In *P. cathemerium* young red blood cells are also much preferred with even normoblasts becoming parasitized (Garnham, 1966).

Factors determining the course taken by erythrocytic merozoites are unknown since even in multiple erythrocyte infections, each parasite may go its own way, becoming a gametocyte of either sex or proceeding to reinvasion of tissue or other erythrocytes. The determining factors most probably lie within each parasite.

B. Stages in the invertebrate host:

The mosquito ingests the mature micro- and macro-gametocytes when taking an infectious blood meal after which development of the gametocytes continues within the blood meal in the midgut of the mosquito. In a process termed gametogenesis, the gametocytes round up and leave their host cells. The micro-gametocytes undergo exflagellation (micro-gametogenesis) during which threadlike flagellated microgametes are extruded from the gametocyte, which will then fertilize the larger spherical macrogametes in the midgut of the mosquito. In the case of *P. relictum*, at a temperature of 21°C, exflaggelation begins within 10 minutes after ingestion of microgametes resulting in 6 or more gametes being extruded. *P. elongatum* microgametes have to be exposed to secretions of the gut of a susceptible mosquito species for exflagellation to occur which would begin in approximately 7 minutes.

Sexual reproduction (fusion of joined gamete nuclei) results in the formation of a fertile zygote, which rapidly differentiates and increases in size to form an elongate, motile form called the ookinete. The ookinetes migrate to and penetrate the midgut epithelial cells and move to the basal lamina of the epithelium (haemocoel side of the

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midgut) where it develops into the oocyst. In *P. relictum* the oocysts start forming 32 – 34 hours after the mosquito has ingested an infective blood meal.

The oocysts undergo asexual reproduction (sporogony) to produce numerous elongate sporozoites. The mature oocysts rupture and release the sporozoites into the haemocoel of the mosquito, which then penetrate the mosquito salivary glands where, during the next blood meal, they will once again gain entry into a new susceptible host. *P. relictum* oocysts are capable of rapid development in the mosquito midgut and at temperatures of 30°C the sporozoites may reach the salivary glands as early as the 4^{th} or 5^{th} day after ingesting an infective blood meal. At lower temperatures, sporogony may be completed in 6 – 8 days. In studies done on African penguins in the Baltimore Zoo, Cranfield *et al.* (1990) found *P. elongatum* sporozoites to appear in the salivary glands after approximately 13 days.

6.3.2.2. Distribution and Species Affected

Avian plasmodia are distributed through most parts of the world with *P. relictum* being the most commonly encountered species and having the widest host range (Garnham, 1966; Bennett *et al.*, 1993; Atkinson, 2001a). The distribution of the different plasmodia coincides with the geographic distribution of their mosquito vectors, which in turn is influenced by environmental conditions (Seed and Manwell, 1977; Atkinson, 2001b).

Although the different *Plasmodium* species infect both domesticated and wild birds, the different *Plasmodium* species within a specific subgenus tend to favour specific avian families. In turn, different avian families are not equally susceptible to *Plasmodium* parasites and therefore, the incidence and pathogenicity of any given *Plasmodium* species, may vary among different host families (Garnham 1966; Atkinson, 2001a).

6.3.2.3. Transmission and Infection

The incidence of *Plasmodium* infections in birds is seasonal due to transmission being dependent on the availability of suitable vector populations and avian malaria can therefore be described as an enzootic disease (Seed and Manwell, 1977; Fix *et al.*, 1988; Atkinson, 2001b).

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The optimal conditions needed for seasonal transmission of avian malaria include ideal environmental conditions, a suitable vector, a source of infection and finally a susceptible host. The role of these conditions in the outcome and impact of *Plasmodium* infections, as well as factors that influence each of these conditions, are as follows (Atkinson and Van Riper, 1991; Atkinson, 2001b):

1) Environmental Conditions

   In areas with a temperate climate, winters are usually cooler which limits the vector populations, but with the onset of warmer weather conditions for transmission become favourable with subsequent increase in vector populations. Vector populations are negatively influenced not only by cold temperatures, but also high temperatures combined with low humidity (Seed and Manwell, 1977). In regions with a warmer climate, *Plasmodium* parasites may be transmitted throughout the year. The environmental conditions are unique for each habitat and ultimately affect the increase in vector populations and subsequent prevalence and transmission of *Plasmodium* parasites.

2) Mosquito Vector

   Mosquito species and even individuals within a species differ in their susceptibility to *Plasmodium* parasites and according to studies done by Huff (1931), this susceptibility seems to be genetic. The development of *plasmodia* within mosquitoes is further influenced by environmental characteristics, of which temperature is probably the most important. However, once susceptible mosquitoes do become infected, they remain infected for life (Seed and Manwell, 1977).

   Due to limited studies on vector epidemiology, the natural mosquito vectors have not been identified for all known incidences of avian malaria. Vectors that have been identified implicate mosquito species belonging to the genus *Culex* to be the usual vectors and thus transmitters of avian *Plasmodium* parasites under natural conditions (Garnham, 1966; Atkinson, 2001a). However, some *Aedes*, *Culiseta* and *Anopheles* species can be infected experimentally (Garnham 1966; Seed and Manwell, 1977). Outbreaks of penguin malaria in a zoo in Antwerp, identified the vector as *Culex pipiens*,

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whilst both *C. pipiens* and *Culex restuans* has been identified as vectors of *P. relictum* and *P. elongatum* infections in African penguins at Baltimore Zoo (Beier and Stoskopf, 1980; Cranfield *et al.*, 1990). Fantham and Porter (1944) identified *Culex fatigans* as the possible vector for avian malaria transmission amongst African penguins at Saldanha Bay on the west coast of the Cape Province in South Africa.

3) Source of Infection

Avian malaria is transmitted from host to host by the bite of bloodsucking mosquitoes that feed on exposed flesh around the eyes, beak, legs and feet and maybe through the webs of the feet (Brossy, 1992). This transmission from one bird to the next is subject to the availability of birds with either a chronic infection or relapse of a latent (low-level) infection as source of *Plasmodium* parasites.

Epidemiological studies done by Beier and Stoskopf (1980) on avian malaria in African penguins at the Baltimore Zoo, indicated surrounding wild summer resident species as well as species of waterfowl to be infected with *Plasmodium* (*P. relictum* and *P. elongatum* identified amongst others) and is therefore a probable reservoir of avian malaria. It is doubtful whether malaria would occur in African penguins in the absence of infected wild birds, since infection in penguins coincide with periods of seasonally high infection rates in wild birds (Beier and Stoskopf, 1980). According to Cranfield *et al.* (1990) there is little potential for spread of infections from penguin to penguin at the Baltimore Zoo and infections rather come from reservoir birds in the Baltimore area. At the San Diego Zoo the origin of infection in penguins is assumed to be birds in the local environment, which are then transmitted to penguins by mosquitoes (Griner, 1974). Infected penguins acting as reservoirs should, however, not be ruled out because if they do become infected, primary infections result in parasitemias which persist for up to 3 weeks (Stoskopf and Beier, 1979). However, once recovered, the penguins are unlikely to serve as carriers of gametocytes for mosquito infections as they rarely exhibit circulating parasites (Beier and Stoskopf, 1980).

If birds do carry a latent infection, a relapse can be induced by stress factors such as nutritional-, environmental- as well as migration stress. At the San

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Diego Zoo, stress in the form of high temperatures and possibly low humidity have been found to be a trigger of malaria deaths in African penguins. Penguins are not affected under moderate weather conditions, but if they are heat-stressed, the existing malaria infections become exacerbated with resulting mortalities (Griner, 1974).

P. relictum has been found to be responsible for avian malaria infections in African penguin populations in the wild (Fantham and Porter, 1944; Brossy, 1992). In the SANCCOB rehabilitation facility, *P. juxtanucleare* has been found to infect penguins during rehabilitation (Grim *et al.*, 2003). The epidemiology of natural avian malaria infections is, however, very poorly documented and further information is required in order to assess the role of avian malaria in the survival of the African penguin in the wild.

4) Susceptible host

All avian species are not equally susceptible to *Plasmodium* infections and factors that might contribute to this variable susceptibility are as follows:

i) Host habitat:

Each habitat has its own unique environmental conditions that can influence host behaviour as well as changes in the vector populations. These influences can delay, advance or affect the intensity of transmission and subsequent infections (Atkinson and Van Riper, 1991).

The African penguins' natural habitat does not favour the proliferation of mosquitoes and according to Brossy (1992), malaria does not normally occur in wild penguin populations off the South African coast. The SANCCOB rehabilitation facility on the other hand, is adjacent to a large shallow lake (Rietvlei) with abundant bird life as well as large numbers of culicine mosquitoes, thereby bringing the penguins close to potential reservoir hosts as well as vectors.

ii) Vector bionomics:

The presence, abundance and feeding habits of suitable vectors within the host habitat influence the prevalence of *Plasmodium* infections (Atkinson, 2001b). In a study done by Beier and Stoskopf (1980) on African penguins housed in an open-air facility at the Baltimore Zoo, it was found that the

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biting cycle of the *Culex* mosquito begins at sunset and ends shortly after sunrise, with peak levels of biting occurring from midnight to 2 am. In the wild, penguins would normally be able to escape the mosquitoes by spending considerable time diving and swimming under water and thus lessoning the chance of becoming infected and at the same time their oily feathers might act as an impediment to biting insects (Fantham and Porter, 1944). At the SANCCOB facility, penguins do not spend the night in water but instead they are huddled in groups in the pens making them easy targets for mosquitoes (Brossy, 1992).

iii) Host behaviour:

Birds are constantly outdoors and are therefore always exposed to possible infection. Studies done by Beier and Stoskopf (1980) on the activity of African penguins in the Baltimore Zoo showed that on average, 85% of the juvenile and 18% of the adult population were outside throughout the night. The larger amount of time spent outside by juveniles compared to adults might be due to social pressure from territorial adults in the indoor nesting area. Although juveniles spend more time outside at night, adult penguins spend adequate time outdoors to enable exposure to infectious mosquitoes (Beier and Stoskopf, 1980).

Birds are also habitual travellers and by migrating to warmer climates in the winter, it is possible for them to be exposed to avian malaria in these warmer climates. Migratory birds can therefore act as reservoirs for *Plasmodium* parasites (Seed and Manwell, 1977). A situation that can be added to this is the non-natural movement of birds. In the case of zoos, it is possible for non-immune individuals to be translocated into an avian malaria endemic area. These relocated individuals, still under stress of adapting to new surroundings, can be exposed to local vector populations and are therefore susceptible to malaria-induced morbidity and mortality (Beier and Stoskopf, 1980; Fix *et al.*, 1988; Atkinson and Van Riper, 1991). At the Baltimore Zoo, avian malaria has been shown to be endemic to the indigenous wild bird populations. Juvenile captive-reared African penguins are exposed to *Plasmodium* infections during their first outdoor exposure resulting in mortalities. If, however, they do survive, they do not

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succumb to malaria for the remainder of their lives (McConkey *et al.*, 1996). A similar situation is seen at SANCCOB where penguins are brought to the mainland for rehabilitation and in the process they are exposed to birds such as the greywing Francolin that can act as a reservoir for *Plasmodium* parasites (Grim *et al.*, 2003).

Another aspect of host behaviour involves breeding habits. Many bird species breed during the spring season when the onset of warmer weather conditions results in an increase in vector populations. The breeding behaviour of birds can therefore lead to the hatching and fledging of non-immune and thus susceptible juvenile birds (Atkinson and Van Riper, 1991; Atkinson, 2001b).

iv) *Innate physiological differences between hosts:*

These differences include factors such as age (young birds are more susceptible than adults), genetic background of the host species and host immune status (Seed and Manwell, 1977; Atkinson and Van Riper, 1991; Atkinson, 2001b). Concurrent diseases such as fowl poxvirus can influence the susceptibility of an avian host to *Plasmodium* infections through immuno-supression, whilst *Eimeria* infections result in an altered immune response (Huff, 1963; Zuckerman, 1977).

v) *Host-Parasite Co-evolution:*

Host-parasite co-evolutionary patterns over time can influence host susceptibility to *Plasmodium* infections (Seed and Manwell, 1977; Cranfield *et al.*, 1990; Atkinson and Van Riper, 1991). *Plasmodium* species seem to be less pathogenic in avian species that have evolved with the parasite, often causing no clinical signs (Anderson and May, 1982; Cranfield *et al.*, 1990; Brooks and McLennan, 1992). In contrast, in those bird species that have not evolved with the parasite, avian malaria can cause varying degrees of pathology and high mortalities. These are species that generally originate from areas with climatic conditions that do not favour the spread of vectors. The African penguin, for example, normally breeds on islands that have very cold, dry or windy conditions (Cranfield *et al.*, 1990).

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According to Lombard *et al.* (1999), wild African penguins carry a clinically insignificant endemic infection of a *P. relictum* subspecies, but if birds are kept in captivity, they contract a different subspecies from flying birds via mosquitoes, with resultant illness. Considering the severity of specifically *P. elongatum* infections in African penguins at the Baltimore Zoo, the rapidly fatal outcome of the disease along with post mortem histological features of penguins that died of *P. elongatum* infections, it might be that African penguins are poorly adapted hosts for *P. elongatum* (Fleischman *et al.*, 1968).

Considering the various morphological and physiological strains of *P. relictum,* and the morphological differences if passages to different hosts are induced, Fantham and Porter (1944) concluded that penguins contain a morphological variety or strain of *P. relictum* for which they proposed the name *Plasmodium relictum* n. var. *spheniscidae*. This *Plasmodium* strain has amongst other histological differences, low extraerythrocytic levels and high intraerythrocytic schizogony, but with a very low level of gametocytes. According to Fantham and Porter (1944), the high intraerythrocytic schizogony levels indicate that parasitism by *Plasmodium* has developed in these birds in early times. It consequently resulted in diminishing pathogenicity and a considerable degree of host tolerance. The parasite can thus maintain itself in the circulating blood without the need for extensive exoerythrocytic schizogony, but due to the very small number of gametocytes, there is poor provision for its transference to new hosts. The parasite may therefore be approaching agametogony, which means that it is incapable of sexual propagation in the mosquito and thus well on its way to self-extermination. According to Laird (1950) this species is invalid and is merely the type strain *relictum* showing a modified appearance due to the unusual host species. Whether or not this subspecies is in fact valid has not been researched further.

6.3.3. PATHOLOGY

The pathology of *Plasmodium* infections can be correlated with the lifecycle and thus different developmental stages of the *Plasmodium* parasite in the host (Atkinson and

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Van Riper, 1991). *Plasmodium* infections typically undergo the following clinical phases (Garnham, 1966; Atkinson and Van Riper, 1991):

i) Prepatent phase

The initial schizogonic cycles of parasites in the tissues when no parasites can be found in the circulating blood cells. The length of this phase will depend on the duration of pre-erythrocytic schizogony, which in turn depends on the parasite species and bird host (Seed and Manwell, 1977).

ii) Acute phase

Parasites start appearing in the circulation and rapidly increase in numbers.

iii) Crisis phase

Parasitemia in the circulating blood cells and physiological stresses reach a peak.

iv) Latent or chronic phase

The host immune system reduces parasitemia to low levels and surviving hosts show little or no obvious signs of infection. The infections thus passes into a latent stage, which may persist for years and possibly even for life. During this period birds can be super-infected with a heterologous strain of the parasite but not a homologous strain. In cases where the bird is able to get rid of the parasite entirely, it immediately becomes susceptible to re-infection, as there might not be any residual immunity.

During acute infections, the onset of clinical signs may be very rapid with infected birds showing signs of lethargy, refusing to eat or drink, appear to be in distress, ruffled feathers, drooped heads and closed eyes (Atkinson and Van Riper, 1991; Atkinson, 2001a). Birds often develop diarrhoea in *P. juxtanucleare* infections (Atkinson, 2001a). The pathological affects of *Plasmodium* infections in birds are associated with both the tissue and erythrocytic stages of infection (Atkinson and Van Riper, 1991).

After sporozoite invasion, the *Plasmodium* parasites develop in tissues of mesodermal origin, cells of the haemopoietic system and cells of the lymphoid-macrophage system (Atkinson and Van Riper, 1991). Schizonts that develop in the endothelial cells lining capillaries of major organs such as the brain and spleen, may

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completely block blood flow to these organs (*P. cathemerium*). Schizonts that preferentially develop in cells of the haemopoietic system (*P. elongatum*) can cause severe anaemia (Atkinson and Van Riper, 1991).

The major pathology in malaria is associated with the blood and the various organs of the circulatory system and the level of cell destruction is usually proportional to the parasitemia level. However, anaemia is by no means always a chief cause of death (Seed and Manwell, 1977). If erythrocytic schizogony occurs, the red blood cells are destroyed either by rupturing during release of merozoites or removal of the infected erythrocytes by cells of the reticuloendothelial system, especially the macrophages of the spleen, liver and bone marrow. This destruction of erythrocytes can lead to severe anaemia (thin and watery blood) if not balanced by the synthesis and release of new erythrocytes (Atkinson, 2001a). Once the infection reaches the erythrocytic stage, gross pathological signs characteristic of *Plasmodium* infections, such as enlargement and discoloration of the liver and spleen, can be seen. The discoloration is due to the accumulation of insoluble malarial pigment in macrophages after phagocytosis of parasites and in heavy infections may turn these organs black (Atkinson and Van Riper, 1991). The malarial pigment, or haematin, is a by-product of haemoglobin digestion and is the iron-containing portion of haemoglobin that the growing parasite cannot metabolize (Seed and Manwell, 1977).

In African penguins pathology and clinical signs of *P. relictum* and *P. elongatum* infections are associated with the exoerythrocytic stages that result in tissue damage (Fix *et al.*, 1988; Cranfield *et al.*, 1990). According to Graczyk *et al.* (1994a) the most severe form of avian malaria with resulting death in African penguins is seen after primary exposure. The second or third malaria episode does not seem to be that fatal. Both *P. relictum* and *P. elongatum* are capable of causing rapidly fatal disease in African penguins (Stoskopf and Beier, 1979). The clinical course of avian malaria, observed amongst African penguins in the Baltimore Zoo, ranged from as few as three hours to three days (Fleishman *et al.*, 1968).

Avian malaria in African penguins may produce non-specific clinical signs and penguins may even die without showing any clinical signs or parasitemia (Griner, 1974). Low-grade parasitemia is especially seen in African penguins infected with *P. elongatum* (Stoskopf and Beier, 1979). Clinical signs that do appear, but only before the bird is near death, include depression, anorexia, lethargy, regurgitation,

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pale mucous membranes and respiratory distress and anoxia (Griner, 1974; Stoskopf and Beier, 1979). Due to the low levels of parasitemia, destruction of the erythrocytes does not cause clinical anaemia in the penguins (Cranfield *et al.*, 1990).

Gross lesions generally considered to be pathognomonic for malaria infections in penguins include an enlarged spleen, swollen liver, congested and extremely oedematous lungs, hydropericardium and epicardial oedema, subcutaneous oedema and fluid collecting in tissues is clear and faintly green (Fantham and Porter, 1944; Fleishman *et al.*, 1968; Griner, 1974; Cranfield *et al.*, 1990; Graczyk *et al.*, 1994a). According to Fleishman *et al.* (1968), the involvement of lungs in the tissue response to *Plasmodium* infections in African penguins (specifically *P. elongatum*) is an unusual manifestation of malaria and may be explained by a difference in the adaption of this parasite in the penguin.

Histological features included exoerythrocytic schizonts in several tissues throughout the body such as the liver, kidneys, brain, spleen, heart and lungs. An acute severe interstitial pneumonia may also be present in the lungs (Fleishman *et al.*, 1968; Griner, 1974; Cranfield *et al.*, 1990; Graczyk *et al.*, 1994a). Peripheral blood smears may or may not show parasitemia (Griner, 1974). Examination of organ smears and tissue secretions in *P. relictum* infections showed marked congestion of capillaries with enlarged erythrocytes and phagocytes whereby passage of normal erythrocytes would appear to have been retarded (Fantham and Porter, 1944). There is, however, not enough destruction seen in the erythrocytes to cause clinical anaemia (Cranfield *et al.*, 1990).

6.3.4. IMMUNITY

Resistance mechanisms of avian species to *Plasmodium* infections are a combination of innate and acquired immunity.

6.3.4.1. Innate (non-specific) immunity

Despite the fact that acute infections in non-immune birds are generally more severe, innate resistance is affected by an interplay of both physiological and biochemical factors that affect the host's susceptibility to parasite infection (Atkinson and Van Riper, 1991).

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Physiological factors include aspects such as host nutrition, nature of physical barriers of the host (skin or membranes of host cells) and age of both the cells that are being infected (erythrocytes) and the host. Young birds usually develop higher and more prolonged parasitemias with a greater mortality than older birds (Seed and Manwell, 1977).

Biochemically, innate immunity is dependent on both humoral factors and phagocytic activity of cells. The humoral factors exert a positive effect in natural resistance, but are of minimal importance compared to the lymphoid-macrophage system, which constitutes a major part of the natural defence of the bird (Seed and Manwell, 1977). Macrophages line the vascular passages of organs actively screening blood and phagocytosing foreign material (both extracellular *Plasmodia* and *Plasmodium*-infected cells). Destruction of parasites does not always occur following phagocytosis, as phagocytic cells themselves serve as host cells for exoerythrocytic parasites (Seed and Manwell, 1977).

6.3.4.2. Acquired (specific) immunity

Acquired immunity develops after exposure of the host to either the infecting pathogen itself or its products via immunization. The type of immunity that develops is basically a T-cell-mediated response with antibodies playing a supportive role (Seed and Manwell, 1977). The protective effect provided by specific antibodies is limited due to the predominantly intracellular habit of the malaria parasites. Those parasites that are released from cells (merozoites or sporozoites) are probably affected by specific antibody while in transit from one host cell to the next. The role of humoral antibodies should, however, not be downplayed. Experimental depletion of plasma- and B-cell populations via neonatal bursectomy rendered birds incapable of mounting a specific humoral response to infection with the avian parasite *P. gallinaceum.* Infections were subsequently more severe with a shorter prepatent period, higher parasitemias and greater mortality rates (Stutz *et al.*, 1972).

The lymphoid-macrophage system with its reparative and anti-parasitic functions is also an important component of the acquired defence mechanism of the host. Early during the primary infection with virulent *Plasmodium* species there is lymphoid cell loss through destructive processes and cell transformation. This is compensated for by a hyperplastic response of the lymphoid tissues. The speed at which this

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recuperative response occurs is vital to the control of infection. If the response is too slow, the bird will succumb (Seed and Manwell, 1977). Super-infected immune birds quickly counterbalance and thus control the initial lymphoid depletion with an intensified hyperplastic response regulated by the spleen and to a lesser extent the liver (Huff, 1963). This immunity depends on a continued low-grade infection due to survival of latent parasites and is termed "premunition" or "infection immunity" (Seed and Manwell, 1977; Sergent and Sergent, 1956). However, resistance conferred by premunition is not re-enforced by repeated re-inoculations, thus the bird is not re-infected by the same strain of parasite. Experimental data indicates that a latent or chronic infection of *P. relictum*, *P. elongatum* or *P. cathemerium* is accompanied by immunity against a super-infection by the same strain of parasite (Sergent and Sergent, 1956).

There are three categories of premunition that can be distinguished (Sergent and Sergent, 1956):

i) Racial or homologous premunition. When, a specific *Plasmodium* parasite strain confers resistance only against itself.
ii) Specific or heterologous premunition. When, a *Plasmodium* parasite strain confers resistance against other strains of the same species.
iii) Generic premunition. Resistance conferred by one species against another species of the same genus.

It is assumed that once birds are infected, they remain infected for life. Manwell (1934) reported on work done on the duration of malarial infections of birds with amongst other, *P. cathemerium*, *P. relictum* and *P. elongatum*. Parasites persisted in the body throughout the period of observation, which was more than one year and up to four years in some studies. He stated that caution must be used in regarding any case of avian malaria as entirely recovered as parasitemia may only be evident during initial stages of infection or during relapses. Parasite levels during chronic stages may be too low for blood smear detection or even too low for sub-inoculations to be successful (Manwell, 1934).

Cranfield *et al.* (1990) tested African penguins in the Baltimore Zoo for persistence of malaria infection and found birds to be positive for malaria for up to eight years. He also reported that if the penguins survive their first infection with avian malaria, their

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immune system appears to be capable of reducing the number of parasites to sub-patent levels.

6.3.5. MALARIA DIAGNOSIS

Early diagnosis of avian malaria is important for efficient treatment of the disease, but the absence of clinical signs can complicate efficient antemortem diagnoses and thus subsequent treatment as it is usually too late for treatment once clinical signs appear (Stoskopf and Beier, 1979; Cranfield *et al.*, 1990). Fleischman *et al.* (1968) demonstrated that the interval between the onset of clinical signs and death in *P. elongatum* infections in African penguins was as short as a few hours. Diagnosis of avian malaria in African penguins on the basis of clinical signs can therefore be inaccurate as penguins are known to die from avian malaria without any premonitory signs. The signs that do develop can be non-specific or confused with other penguin diseases, such as aspergillosis and bacterial gastroenteritis (Stoskopf and Beier, 1979).

Stoskopf and Beier (1979) suggested the use of haematological parameters as an indicator of avian malaria infections in African penguins. The haematological parameters include total white blood cell counts and relative lymphocytosis. Graczyk *et al.* (1994d) found these haematological parameters to be valid indicators of high-risk birds, but they are not sensitive enough (Cranfield *et al.*, 1990). Fix *et al.* (1988) found no correlation between malaria infections and haematological changes in Magellanic penguins.

Graczyk *et al.* (1995c) evaluated the use of certain serum chemistry parameters as a diagnostic tool for avian malaria in African penguins. The tissue phase of *Plasmodium* infections occurs before the blood stage and is the phase that causes disease and death. It was therefore thought that serum chemistry would give some insight for diagnosis (Cranfield *et al.*, 1990). Values obtained for gamma-glutamyltranspeptidase (GGTP), alanine aminotransferase (ALT), alkaline phosphatase, creatinine, uric acid, triglyceride, phosphates and very light-density lipoprotein can be used to indicate possible exposure to avian malaria, but only ALT, GGTP and creatinine were of diagnostic value being able to indicate the clinical malaria status of African penguins. However, factors such as blood collection, storage and processing can affect serum chemistry variables, leading to misdiagnosis (Graczyk *et al.*, 1995c).

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Cranfield *et al.* (1990) studied the use of the quantitative buffy coat tube that is used for detection of human malaria. Parasite infected red blood cells are lighter than normal red blood cells causing them to collect at the top of the red cell layer in the haematocrit tube. A stain is added causing the human parasite to fluoresce. In the case of penguin blood, fluorescing intercellular particles were found in both infected and control animals. The fact that the erythrocytes are nucleated may have added to obscuring the viewing of the parasite.

Following exposure, parasitemia can be observed by examining Giemsa-stained thin blood smears (Atkinson and Van Riper, 1991). Romanowski-, Wright- or Leishman's stains can, however, also be used (Seed and Manwell, 1977). *Plasmodium* infections are quantified as the number of parasites or infected erythrocytes per 1 000 – 10 000 uninfected erythrocytes (Atkinson and Van Riper, 1991). Using this technique, both asexual (trophozoites and schizonts) and sexual stages (gametocytes) can be identified in the erythrocytes. However, blood smears are time consuming and in order to detect parasitemias as low as 0.001% in penguin blood smears, the smears should be of good quality and well stained. In addition to this, a minimum of 40 000 erythrocytes should be examined to be able to detect parasitemias this low (Stoskopf and Beier, 1979). In recovering birds, the parasitemia levels may drop to levels where no parasites can be demonstrated in the blood. Even in birds with a high level of exoerythrocytic infection, parasitemia may be too low for detection with birds dying before parasitemia is found (Herman *et al.*, 1966; Cranfield *et al.*, 1990). If death occurs prior to invasion of circulating blood cells, diagnosis is complicated and dependent on histopathological examination of Giemsa-stained impression smears of fresh tissue organs (Atkinson and Van Riper, 1991). African penguins with a *P. elongatum* infection usually have a low peripheral parasitemia due to *P. elongatum* exoerythrocytic forms being more pronounced and causing extensive pathological changes (Fleischman *et al.*, 1968). This low level of parasitemia for *P. elongatum* infections was confirmed by Stoskopf and Beier (1979) and found to be usually less than 0.01%.

An alternative method for diagnosis of avian malaria, in cases where parasitemia levels are too low for blood smears, is isodiagnosis (Cranfield *et al.*, 1990). This technique involves taking infected penguin blood and inoculating it into a previously unexposed susceptible host (usually day-old ducklings) (Manwell and Hatheway,

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1943). The initial course of parasitemia is reproduced in the ducklings thereby magnifying the infection resulting in high parasitemia. Ducks can be infected experimentally with *Plasmodium* parasites, but they overcome the infection and do not die from it (Cranfield *et al.*, 1990). Blood of the ducklings was examined periodically using thin blood smears. This technique was shown to be useful in determining the prevalence of *Plasmodium* in populations with an unknown history of exposure to infection as well as identifying the infecting *Plasmodium* parasites (Herman *et al.*, 1966; Stoskopf and Beier, 1979). This technique, however, takes 4 – 5 days, has a fair amount of expense and there might be cases where parasitemia levels are even too low for isodiagnosis (Cranfield *et al.*, 1990). Due to the time factor, results may not be available before the affected bird dies (Stoskopf and Beier, 1979).

The ELISA test has been shown to be a useful and sensitive diagnostic tool to indicate exposure to *P. relictum* and *P. elongatum* parasites in African penguins as well as various other penguin species (Graczyk *et al.*, 1994c; Graczyk *et al.*, 1995b; Graczyk *et al.*, 1995d). Although humoral antibodies are only partly responsible for protection it is assumed that they will reflect the amount of cellular immunity developed.

6.3.6. PREVENTION AND CONTROL

Pharmacokinetics of anti-malarial drugs has not been determined in penguins and dosages are usually extrapolated from other species (Fix *et al.*, 1988). Stoskopf and Beier (1979), as well as Graczyk *et al.* (1994d), based their therapeutic regimen for African penguins in the Baltimore Zoo on standard human infant treatment schedules. Due to the short period (few hours) that can elapse between the onset of clinical signs and death, treatment was started as soon as parasitemia was detected. The treatment starts with chloroquine phosphate that acts against the erythrocytic stage thereby eliminating erythrocytic parasites (Fix *et al.*, 1988; Graczyk *et al.*, 1994d). This is followed by a lower dose of chloroquine together with primaquine phosphate. Primaquine acts against the exoerythrocytic stages of the parasite and has been shown to inhibit mitochondrial respiration of the primary and secondary liver stages of human malarial parasites (Fix *et al.*, 1988; Cranfield *et al.*, 1994). Fix *et al.* (1988) also used this treatment for Magellanic penguins. Birds were cleared from any observable parasitemia, but the treatment failed to free the penguins of all

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malaria parasites. This can, however, be seen as an advantage if immunity is in fact based on premunition as a result of chronic infections (Stoskopf and Beier, 1979; Graczyk *et al.*, 1994d).

Although according to Brossy (1992), primaquine may not be the ideal drug for prophylaxis in penguins, he nevertheless suggested that SANCCOB penguins should be given prophylaxis as a preventative measure because some birds remain healthy despite infections. It is thus important to treat birds so that they do not become a source of infection for the rest of their colony. Currently anti-malaria treatment of penguins at SANCCOB consists of both primaquine and chloroquine (Darmal®). This is supplemented with Essentiale® (vitamin supplement) to maintain optimum hepatic function, as the above drugs can damage the liver. Other drugs that are given depending on the condition of the bird includes Valium®, for the suppression of convulsions (which are sometimes a sign of malaria), Lasix®, which is a diuretic and is used to clear fluid off the lungs or heart and finally Millophyline®, which is a cardiac and respiratory stimulant that helps ease their breathing and open the bronchi so they can breathe easily in cases where the lungs become filled with fluid. Generally, if a penguin needs both Lasix and Millophyline, it is usually too late to save the bird (Parsons, 2003).

Due to possible contra-indications to long-term prophylatic treatment it may, however, not be possible to preventatively treat captive penguins in zoos (Brossy, 1992). Avian haemoprotozoans are very resistant to antibiotics and their routine use will only be justified if extensive trials show them to be effective (Brossy, 1992). Cranfield *et al.* (1994) showed naturally acquired parasites in African penguins to survive chloroquine and primaquine therapy and once the penguins are treated with an immunosuppression drug the parasites recrudesce into the erythrocytes. It is likely that either infected erythrocytes persist in the deep vascular sites in penguins or, dormant sporozoites and pre-erythrocytic forms of malarial parasites survive in the endothelial tissues of African penguins. Whatever the case may be, it is clear that basing anti-malarial therapy on human infant treatment schedules is not adequate to eliminate the exoerythrocytic stages of the *Plasmodium* parasite in African penguins (Cranfield *et al.*, 1994).

Besides prophylaxis, the control of avian malaria can be achieved by reducing transmission from infected to susceptible birds by trying to reduce or eliminate

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mosquito populations. This, however, can be very difficult and would involve effective habitat management involving a combination of screening birds as well as using effective mosquito repellents or pesticides (Brossy, 1992; Atkinson, 2001b). However, due to the high susceptibility of the African penguin to avian malaria parasites and the high mortality seen in juveniles, captive penguins should preferably not be exhibited outside where they can be exposed to mosquitoes.

Indoor facilities on the other hand should also be maintained mosquito-free to prevent the possibility of parasite transmission (Graczyk *et al.*, 1994d). This might be achieved by housing them in a cool environment that excludes all insect vectors such as the San Diego Zoo where they experimented with a moving curtain of air passing across the penguin exhibit thereby discouraging mosquitoes from entering and resting on exposed surfaces (Griner, 1974).

At SANCCOB, the efficient management and control of avian malaria requires knowledge regarding the epidemiology of avian malaria in African penguins. This includes determining the immunity levels of penguins to avian malaria on entry and during the rehabilitation process. These aspects will be addressed in the next chapter.

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CHAPTER 7:

Detection of anti-*Plasmodium* antibody levels in African penguins admitted to a rehabilitation facility

7.1. INTRODUCTION

It has been estimated that the present population numbers of the African penguin in the wild would have been 19% lower had oiled and injured birds not been rehabilitated (Nel *et al.*, 2003). Thus SANCCOB has, through its rehabilitation efforts, played an important role in the conservation of the African penguin. During rehabilitation at SANCCOB significant mortalities due to avian malaria are incurred. A reduction in these mortalities would not only make the rehabilitation process more efficient, but this would also bring about an even greater increase in the African penguin population in the wild. At the same time, however, the rehabilitation process might be responsible for introducing avian malaria into these wild penguin populations with according detrimental effects. Thus, efficient control of avian malaria in African penguin during rehabilitation may have important implications for the survival of the species. An important aspect influencing the epidemiology of this disease is the immune competence of the birds. If this can be improved, survival rates may be improved and infection rates reduced during rehabilitation.

The objectives of this study were therefore:

1) To develop an ELISA for the specific detection of anti-*Plasmodium* antibodies in African penguins.
2) To determine the effect of anti-*Plasmodium* antibody production on the survival of penguins during rehabilitation.
3) To determine the effect of oiling on the ability of penguins to produce an anti-*Plasmodium* antibody response.
4) To determine whether birds contract avian malaria at SANCCOB.

The results of this study are described at the end of this chapter in the form of an independent manuscript. The manuscript is preceded by background information

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regarding the ELISA development as well as the approach used for sample collection and processing for ELISA determination of anti-*Plasmodium* antibody levels.

7.2. DEVELOPMENT OF AN ANTI-*PLASMODIUM* ELISA

Graczyk *et al.* (1993) used the ability of anti-*P. relictum* and anti-*P. elongatum* antibodies to immunologically cross-react with the human parasite *P. falciparum* antigens as the basis for the development of an ELISA for the detection of anti-*P. relictum* and anti-*P. elongatum* antibodies in ducklings. In this ELISA, a recombinant circumsporozoite (CS) protein named $R32tet_{32}$ was used as capture antigen. The protein sequence consisted of the repeating region of the CS-protein of *P. falciparum* that contains a 30 tetrapeptide repeat sequence of Asn-Ala-Asn-Pro and two tetrapeptides with sequence Asn-Val-Asp-Pro (Young *et al.*, 1985).

Graczyk *et al.* (1994c) later used this $R32tet_{32}$ protein as capture antigen in an ELISA for detecting and monitoring levels of anti-*Plasmodium* species in both African (Graczyk *et al.*, 1995a; Graczyk *et al.*, 1995d), and other penguin species (Graczyk *et al.*, 1995b). This capture antigen was also successfully used in an ELISA for determining the transfer of maternal antibodies in African penguins (Graczyk *et al.*, 1994b; Graczyk and Cranfield, 1995).

Instead of using a recombinant CS-protein as capture antigen, Ballou *et al.* (1985) on the other hand, synthesized regions of the CS-protein of *P. falciparum* for the purpose of determining appropriate vaccine targets. Antibodies produced against the repeating regions of these synthetic peptides, recognized native CS-protein thereby blocking sporozoite invasion. This supported the feasibility of using synthetic peptides of the repeating region for vaccine development.

Due to the fact that antibodies raised against synthetic peptides were able to recognise the native protein as well as the known cross-reactivity between anti-*P. relictum*, anti-*P. elongatum* and anti-*P. falciparum*, it was decided to make use of a synthetic peptide from the CS-protein repeating region of *P. falciparum* as capture antigen in an ELISA for the detection of anti-*Plasmodium* parasites in African penguins at SANCCOB.

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7.2.1. *P. FALCIPARUM* CS-PROTEIN AS CAPTURE ANTIGEN

The first stage of malaria infections includes sporozoites, which develop in and are injected by mosquitoes into the vertebrate host (Dame *et al.*, 1984). This sporozoite stage has been shown to be highly immunogenic. By inoculating susceptible hosts with ultraviolet radiation-attenuated sporozoites, a protective immune response is induced (Yoshida *et al.*, 1980; Cochrane *et al.*, 1982). This holds considerable potential for vaccine development and thus prevention of the spread of infections. By eliminating this sporozoite stage, the development of subsequent stages responsible for host disease as well as stages that can be transmitted to other hosts via mosquitoes (gametocytes), can be prevented (Dame *et al.*, 1984). However, these sporozoites are in limited supply as they can only be found in the mucous secretions of mosquitoes, making the isolation of large amounts of sporozoites very difficult. This along with the instability of isolated sporozoites makes the use of irradiated sporozoites impractical (Dame *et al.*, 1984). Studies were therefore undertaken to identify the relevant antigenic determinants that could then be used as possible malaria vaccines (Young *et al.*, 1985).

Studies done in different mammalian hosts using monoclonal antibodies (mAb) identified the protective antigens to be the CS-proteins that are uniformly distributed over the entire surface of the sporozoite membrane (Potocnjak *et al.*, 1980; Cochrane *et al.*, 1982; Nardin *et al.*, 1982). These antigens are stage specific and can only be found in mature sporozoites in the salivary gland of infected mosquitoes (Santoro *et al.*, 1983). Similarly, a CS-protein was also found in malaria sporozoites of the avian parasite *P. gallinaceum* (Krettli *et al.*, 1988). In the case of the avian parasite *P. gallisepticum*, these antigens have also been found on the sporozoites in the oocyst (Krettli *et al.*, 1988).

The complete CS gene of the human malaria parasite *P. falciparum* was subsequently cloned and sequenced by Dame *et al.* (1984). The sequence of this gene was shown to be typical of a membrane protein with an NH_2-terminal signal peptide and a COOH-terminal anchor domain. An interesting characteristic structural feature of the CS-protein is the presence of a large central repeat domain, which in the case of *P. falciparum* contains 41 tandem repeats of a tetrapeptide, 37 with the sequence Asn-Ala-Asn-Pro, interspersed with four tetrapeptides with the sequence Asn-Val-Asp-Pro. In fact, this type of repeating amino acid sequences is also seen in

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other malarial protein antigens (Coppel *et al.*, 1983). This repeating epitope has subsequently been shown to be the major immunogen of the CS-protein recognized by mAb (Dame *et al.*, 1984; Zavala *et al.*, 1983).

All *Plasmodium* species studied to date were found to contain an analogous set of CS-proteins on their sporozoites. The fact that mAb recognise these antigens and can subsequently abolish parasite infectivity, suggest a common function for these proteins that is essential for development of the malaria parasite in its vertebrate host (Santoro *et al.*, 1983). Studies done by Hollingdale *et al.* (1982) on the rodent malaria *P. berghei*, indicate a role of the CS-proteins in target cell adhesion by the parasite. Despite the overall structural similarity between CS-proteins of different *Plasmodium* species there is limited sequence homology over most of the protein (Dame *et al.*, 1984). The greatest sequence homology in all mammalian *Plasmodium* parasites is seen within the two short regions either side of the repeat domain designated region I and region II-plus (Dame *et al.*, 1984; Sinnis *et al.*, 1994). Adding the avian parasite *P. gallinaceum* to this comparison reveals an altered region I whilst region II-plus remains similar. This suggests a similar function in both mammalian and avian sporozoites for region II-plus, whilst region I may be involved in specificity of invasion (McCutchan *et al.*, 1996). In a comparison of the CS-protein sequence of *P. gallinaceum* with that of other mammalian parasites, McCutchan *et al.* (1996) found that the repeat region could not be aligned and only the N- and C-terminal regions could be unambiguously aligned.

Differences in the functional domains between avian plasmodia and the human parasite *P. falciparum* might be a reflection of biological differences. Avian sporozoites develop in culicine mosquitoes and initially infect and develop in macrophages in the avian host, whilst mammalian malarias develop in anopheline mosquitoes and infect and develop in hepatocytes in the mammalian host (McCutchan *et al.*, 1996).

Despite the known species specificity and differences seen between CS-protein sequences of different *Plasmodium* species, there is some immunological cross-reactivity. Some mAb produced against *P. knowlesi* (simian) cross-reacted with sporozoites of *P. cynomolgi* (simian) and *P. falciparum* (Cochrane *et al.*, 1982). Similarly, mAb to the repeating epitope of *P. falciparum* reacted with *P. berghei* sporozoites (Young *et al.*, 1985), and mAb produced against *P. gallinaceum* reacted

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with sporozoites of *P. berghei* (Krettli *et al.*, 1988). This ability of antibodies to cross-react, despite the obvious differences in primary protein sequence, might be due to structural and functional similarities of the secondary and tertiary structures resulting in common conformational epitopes (Cochrane *et al.*, 1982; Dame *et al.*, 1984).

Phylogenetic studies using the small-subunit ribosomal RNA gene sequences (Waters *et al.*, 1991) and CS-protein sequences (McCutchan *et al.*, 1996), as well as structural (Sinden *et al.*, 1978) and nucleotide composition studies (McCutchan *et al.*, 1984) indicated a close evolutionary relationship between avian *Plasmodia* and *P. falciparum*. This close evolutionary relationship between avian *Plasmodia* and *P. falciparum* is further illustrated in the ELISA developed by Graczyk *et al.* (1993) where anti-*P. relictum* and anti-*P. elongatum* antibodies cross-reacted with *P. falciparum*. Whether *P. falciparum* arose directly from an avian *Plasmodium* or whether there is a common ancestor, is still uncertain (Waters *et al.*, 1991).

7.2.2. SYNTHETIC PEPTIDE AS CAPTURE ANTIGEN

Ballou *et al.* (1985) synthesized peptides from the CS-protein repeat region of *P. falciparum* containing 8-, 10-, 12-, 14- and 16 residues of the repeat sequence NANP. Antisera were raised to these peptides and subsequently tested by indirect immunofluorescent assay for cross-reactivity with human, primate, rodent and avian malaria sporozoites. This cross-reactivity was more pronounced with the 16 residue peptide antisera.

To maximize the cross-reactivity of malaria parasites in African penguins to *P. falciparum*, it was decided to use a 16 amino acid peptide corresponding to the repeating region of the CS-protein of *P. falciparum* as capture antigen in this study. The 16 amino acid peptide, with the sequence $(NANP)_4$, was synthesized by the BIOPEP Peptide Synthesis Laboratory at the Biochemistry Department, University of Stellenbosch.

Prior to use, the peptide was conjugated to BSA. The use of a protein carrier, such as BSA, to immobilize the peptide antigen on the polystyrene surface of the mictrotitre plate, allows for a more rigid attachment of the peptide antigen. Adsorption of peptide directly to the surface is often insufficient resulting in desorption of antigen during incubation and wash steps. The adsorption of protein on the other hand is usually more effective (Ivanov *et al.*, 1992). The procedure used for conjugation of

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BSA to the synthetic peptide consisted of the following three reaction steps and is shown in Figure 7.1:

1) *Introduction of maleimide groups into BSA:*

This was accomplished by the reaction of the primary amine groups of BSA with the N-hydroxysulfosuccinimide (NHS)-ester group of sulfosuccinimidyl 4-[N-maleimidomethyl]-cyclohexane-1-carboxylate (Sulfo-SMCC, Pierce). To this end, BSA was dissolved at a concentration of 5 mg/ml in a buffer containing 25 mM HEPES and 1 mM EDTA (pH 7.8). To each 1 ml BSA solution, 30 µl Sulfo-SMCC (60 mg/ml) was added while mixing gently for 90 min in a dark room at room temperature (RT). Free Sulfo-SMCC was removed by dialyzing overnight at 4°C with a buffer containing 25 mM HEPES and 1 mM EDTA (pH 6.8). Maleimide groups incorporated into the BSA were determined by firstly incubating the activated BSA with 2-mercaptoethanol for 25 min at RT and secondly, determining the remaining thiol groups by adding Aldritiol (Sigma) and measuring the absorbance at 324 nm after a 2 min incubation at RT. The activated BSA was stored at -20°C until used (no longer than four weeks). The ratio of maleimide groups incorporated per BSA molecule was subsequently calculated as being 8:1.

2) *Introduction of a sulphydryl group into the malaria peptide:*

The malaria peptide was synthesized containing an S-acetylmercaptosuccinic anhydride (SAMSA) group coupled to the peptide's primary amine group. A free sulfhydryl group was generated from the SAMSA-peptide conjugate by cleaving off the acetic acid group using 0.5 M hydroxylamine (3.45% hydroxylammonium chloride, 18.56% triethanolamine hydrochloride, 0.2% 0.5 mM EDTA, pH 7.0).

3) *Conjugation of peptide to activated BSA:*

The SAMSA-peptide conjugate, dissolved in dry dimethylsulfoxide (DMSO), was added to the activated BSA in a 1:1, 3:1, and 9:1 ratio. For each 1 ml of BSA solution, 50 µl 0.5 M hydroxylamine (pH 7.0) was added after which the solution was mixed on a gyro-rocker overnight at RT. Conjugation was stopped by firstly adding 2-mercaptoethanol and incubating the solution for 20 min at RT. Secondly, N-ethylmaleimide (NEM, Sigma) was added after which the solution was incubated again for 15 min at RT. The conjugate was dialyzed overnight against a

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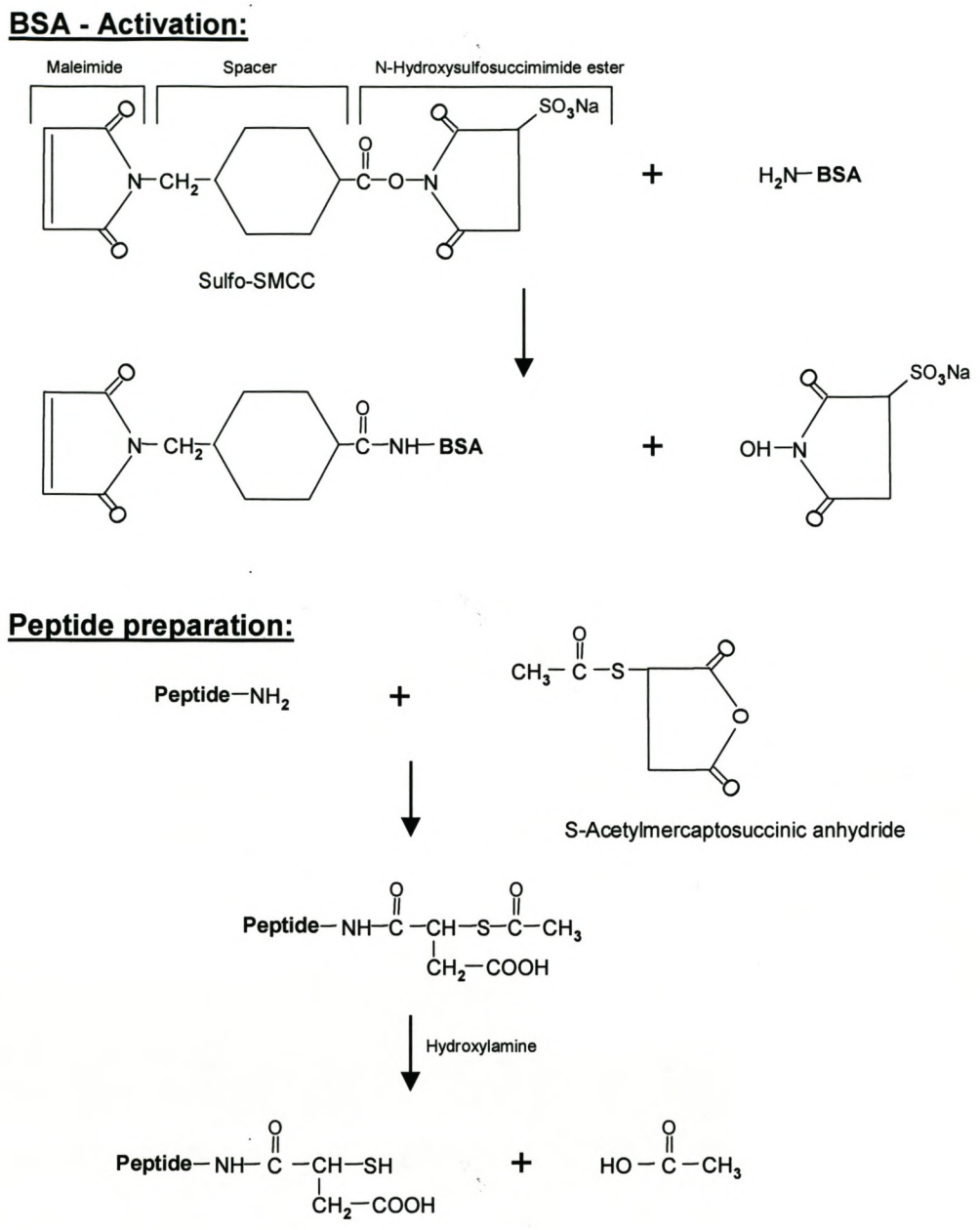

Figure 7.1: Conjugation of the *Plasmodium* peptide to BSA

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buffer containing 25 mM HEPES and 1mM EDTA (pH 6.8) after which aliquots were made and stored at -80°C.

By using different ratios of malaria peptide to maleimide derivatized BSA (1:1, 3:1 and 9:1) three conjugates of different malaria peptide to BSA ratios were generated. Although this ratio does not necessarily reflect the actual coupling ratio, these conjugates were referred to as 1:1, 3:1 and 9:1 malaria peptide-BSA conjugates.

Assuming that there was no losses or dilution of the conjugate during the final dialysis, the total volume of each of the 1:1, 3:1 and 9:1 conjugates was calculated by adding the following volumes: volume of activated BSA used for conjugation, volume of peptide dissolved in DMSO, volume of hydroxylamine, volume of 2-mercaptoethanol and volume of NEM. The concentration (mg/ml) of the malaria peptide-BSA conjugate was calculated by dividing the mass (mg) of the peptide (which was originally dissolved in DMSO and added to the activated BSA) by the total volume of the conjugate after dialysis (ml) as calculated above. The concentration was calculated for each of the different peptide-BSA conjugate ratios.

7.2.3. OPTIMIZATION OF ANTI-*PLASMODIUM* ELISA

The synthesized *Plasmodium* peptide-BSA conjugates were subsequently used as coating antigens in an ELISA to determine penguin anti-*Plasmodium* antibody levels. In order to determine optimal coating conditions, these conjugates of different peptide-BSA ratios (1:1, 3:1 and 9:1) were tested at different concentrations. The test sera used during optimization of the ELISA were obtained from penguins that were confirmed as being malaria positive on the basis of a bloodsmear. Three test sera were used, one giving low absorbance values in the penguin anti-*Plasmodium* ELISA, one giving a medium absorbance value and one giving a high absorbance value. The ELISA titres obtained for each of the control sera using different peptide-BSA ratio conjugates at different coating concentrations are shown in Figure 7.2A, B and C. In Figure 7.2A absorbance values increased with an increase in peptide-BSA ratio, but reached a plateau, or started decreasing, at a higher than 3:1 peptide-BSA ratio. At the same time an increase in absorbance values was seen with an increase in coating concentration. A plateau was, however, reached after a 0.5 µg/ml coating concentration. In Figure 7.2B a medium titre serum was used. Absorbance values once again increased with increasing peptide-BSA ratio conjugates, but started

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decreasing at a higher than 3:1 peptide-BSA ratio and at the same time absorbance values started decreasing with coating concentrations higher than 0.5 µg/ml. In Figure 7.2C a high titre serum was used and here the absorbance values either decreased, or reached a plateau, at a 3:1 peptide-BSA ratio. If coating concentrations lower than 0.5 µg/ml were used, inconsistent absorbance values were obtained. On the basis of these results it was decided to use the 3:1 peptide-BSA ratio conjugate at a coating concentration of 0.5 µg/ml in the penguin anti-*Plasmodium* ELISA.

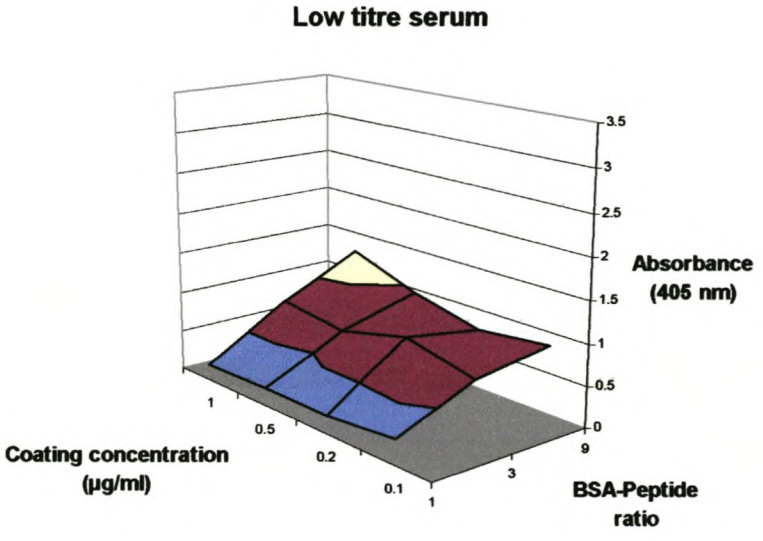

Figure 7.2A: The coating concentration versus peptide-BSA ratios of a serum with low absorbance values in the penguin anti-*Plasmodium* ELISA.

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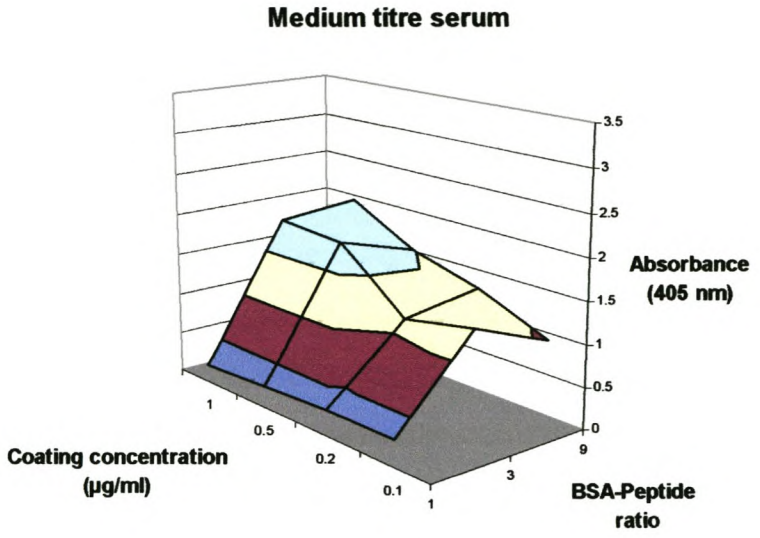

Figure 7.2B: The coating concentration versus peptide-BSA ratios of a serum with medium absorbance values in the penguin anti-*Plasmodium* ELISA.

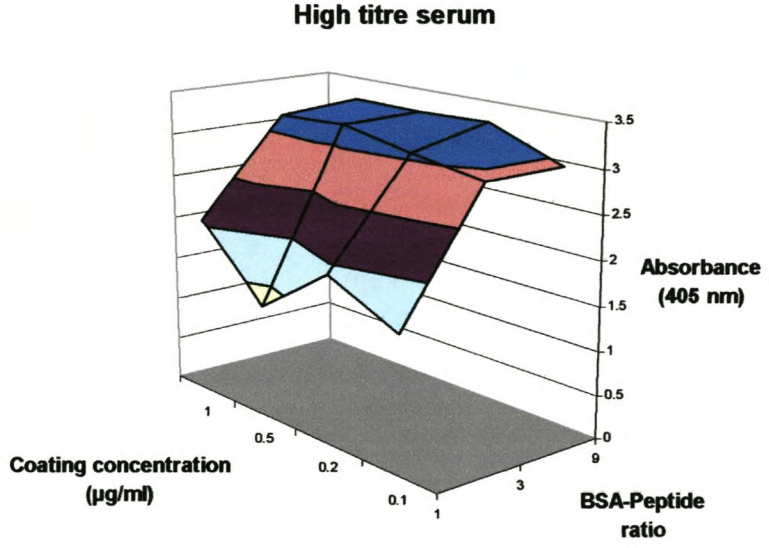

Figure 7.2C: The coating concentration versus peptide-BSA ratios of a serum with high absorbance values in the penguin anti-*Plasmodium* ELISA.

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7.3. SAMPLE COLLECTION AND PREPARATION

Blood samples from a total of 30 penguins present in the SANCCOB rehabilitation facility were obtained on a weekly basis for a period of 15 months. Financially the prolonged existence of the SANCCOB facility relies on the generosity of donors and with a very small number of permanent staff, the help of volunteers is very much needed for the day-to-day running of the facility. Sample collection was therefore done in a manner so that it would firstly bring about minimal additional work and samples were collected along with routine blood samples taken weekly for malaria blood smears. Secondly, it was important not to incur additional costs to the rehabilitation procedures at SANCCOB. This meant that the release of apparently healthy birds was not delayed just because they were in this trial, resulting in a difference in the number of ELISA titre values obtained for each bird. Consequently, this resulted in missing data values for those birds that were released before they had spent four weeks in the facility. Released birds were, however, replaced so that 30 penguins were always sampled at any given time, unless fewer birds were actually being rehabilitated.

Blood samples were collected as described by Graczyk *et al.* (1994c), except that only a single 50 μl blood volume, as measured by calibrated heparinized capillary tubes, was deposited on filter paper strips (Immunovet Services) and air dried. The collection of blood samples on filter paper was found to be a convenient way of obtaining blood from the penguins without adding additional stress to the rehabilitation process. Filter paper samples have the further advantage of easy handling and storing of a large number of sera and most importantly, it allows for convenient transport. After collection of samples, filter papers could easily be sent to Stellenbosch by post without the need for costly overnight courier facilities.

According to Graczyk *et al.* (1993), storage of serum samples on filter paper does not influence the antibody binding capacity. In this study, however, the storage conditions of blood samples on filter paper were found to be crucial. In a preliminary trial, six filter paper samples were collected from a single penguin at the same time, sent to Stellenbosch by post and stored at room temperature in a dark envelope. One sample was tested immediately after arrival (Week 1) using the anti-malaria ELISA and the other filter paper samples tested with one-week intervals thereafter. Results showed the antibody levels to drop with each successive week (Table 7.1). For this

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reason, it was decided that filter paper samples should preferably be sealed in such a way as to minimize oxygen contact and stored at -20°C until use.

Table 7.1: Influence of storage of filter papers on antibody titres

WEEKS	AVERAGE ELISA TITRE*
0	0.499
1	0.178
2	0.055
3	0.015
4	0.031
5	0.021

* Average of 3 ELISA titre values

7.4. DETECTION OF ANTI-*PLASMODIUM* ANTIBODY LEVELS IN AFRICAN PENGUINS ADMITTED TO A REHABILITATION CENTRE

7.4.1. CONTRIBUTIONS OF CO-AUTHORS

The following manuscript contains the original and independent work of the author of this dissertation. The author performed all experimental work, except for the immunization of rabbits. The contribution of each of the co-authors was as follows:

Dr. N. Parsons is the veterinarian at the SANCCOB rehabilitation facility in Tableview, Cape Town. She was involved in the development of this project and was responsible for sample collection. All bloodsmear data was obtained from her.

Ms. E. van der Merwe was the SANCCOB centre manager and was involved in the initial planning and development of this project.

Prof. D.U. Bellstedt is an associate professor of the Department of Biochemistry at the University of Stellenbosch, and as promoter of this dissertation he was involved in all aspects of development and execution of this study. He was responsible for all collaborations with SANCCOB as well as obtaining funding from the World Wildlife Fund for this project.

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DETECTION OF ANTI-*PLASMODIUM* ANTIBODY LEVELS IN AFRICAN PENGUINS ADMITTED TO A REHABILITATION CENTRE

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ABSTRACT

The African penguin is the only penguin species that breeds in Africa and populations are currently about 10% of that at the start of the 20th century (Nel *et al.*, 2003). Oil spills, affecting thousands of birds, are seen as an important immediate threat to African penguin populations. The South African Foundation for the Conservation of Coastal Birds (SANCCOB) is a seabird rescue and rehabilitation centre located at Tableview in Cape Town and is largely dedicated to the rehabilitation of diseased, injured or oiled penguins. Despite the success of SANCCOB in rehabilitating penguins, significant mortalities due to avian malaria do occur at the facility. The aim of this study was to develop an anti-*Plasmodium* enzyme-linked immunosorbent assay (ELISA) in order to assess the natural immunity of penguins to avian malaria on entry into the SANCCOB facility and during the rehabilitation process. Results indicated significant increases in anti-*Plasmodium* antibody levels after admission during summer months. Infection with malaria and not parasite recrudescence was viewed to be the cause of this increase, as increases in immunity did not occur during the winter months when vectors were abscent. It thus appears that penguins are exposed to avain malaria during rehabilitation and repositioning of the SANCCOB facility should be considered. The effect of oiling on reducing immunity during the rehabilitation period was also assessed, but no significant effects were found.

INTRODUCTION

The African penguin is the only penguin species that breeds in Africa and populations are currently about 10% of that at the start of the 20th century (Nel *et al.*, 2003). A

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decrease in population size of the African penguin as well as being concentrated within a relatively small geographic area makes them more vulnerable to any event that can trigger further decline (Stoskopf, 1993). Therefore, events such as oil spills, affecting thousands of birds, are seen as an important immediate threat to African penguin populations. The South African Foundation for the Conservation of Coastal Birds (SANCCOB) is a seabird rescue and rehabilitation centre located at Tableview in Cape Town and is largely dedicated to the rehabilitation of diseased, injured or oiled penguins. A large percentage of penguins that have been rehabilitated and released not only survive well, but they return to their own colonies, former nests and mates and continue to breed successfully. Despite the success of SANCCOB in rehabilitating penguins, significant mortalities due to avian malaria do occur at the facility (Brossy, 1992). Avian malaria is a known cause of mortalities in captive penguins kept in open-air facilities (Fleischman *et al.*, 1968; Griner, 1974; Stoskopf and Beier, 1979; Cranfield, 1990), as is the case at SANCCOB. In the wild, penguins would normally be able to escape mosquito vectors by spending a considerable time in the water and thus lessening the chance of becoming infected. The SANCCOB rehabilitation facility is adjacent to a large shallow freshwater lake (Rietvlei) with abundant bird life as well as large numbers of culicine mosquitoes, thereby bringing the penguins close to potential avian malaria reservoir hosts as well as vectors.

It is assumed that penguins do not harbour avian malaria in the wild, but that they become parasitized after arrival in captivity (Fleishman *et al.*, 1968; Beier and Stoskopf, 1980; Fix *et al.*, 1988). The possibility of avian malaria being contracted by penguins at the rehabilitation facility causes concern as to the dangers of introducing avian malaria into already endangered wild populations. In the facility, early diagnosis of avian malaria is important for efficient treatment of the disease, but the absence of clinical signs can complicate efficient diagnosis and thus subsequent treatment as it is usually too late for treatment once clinical signs appear. Currently, parasitemia at SANCCOB is monitored using thin blood smears. Serious pathological effects and even death can occur prior to the appearance of parasites in the circulating blood cells making the diagnosis of *Plasmodium* infections difficult by ordinary blood smears (Atkinson and Van Riper, 1991). The lack of parasites in the erythrocytes does not exclude their possible presence in the endothelium or heamopoietic tissue (Cranfield *et al.*, 1990).

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On the basis of cross-reactivity between *P. relictum*, *P. elongatum* and *P. falciparum* antibodies, Graczyk *et al.* (1993) developed an ELISA for the detection of anti-*P. relictum* and anti-*P. elongatum* antibodies using a recombinant circumsporozoite (CS) protein of *P. falciparum* as capture antigen. Graczyk et al. (1994c) evaluated this ELISA for the diagnosis of avian malaria infections in African penguins at the Baltimore Zoo and found it to be an effective tool for diagnosing exposure to avian malaria in penguins.

The objectives of this study were firstly to establish whether the ability of penguins to produce an anti-*Plasmodium* antibody response influences their survival rate. In order to determine these immune responses, an anti-*Plasmodium* ELISA was developed for this purpose. Secondly, the main function of SANCCOB is the rehabilitation of oiled penguins and therefore it was important to determine whether oiling influences the penguins' ability to produce an anti-*Plasmodium* immune response. Finally, it was determined if penguins became infected with *Plasmodium* during their rehabilitation at SANCCOB by measuring the natural anti-*Plasmodium* antibody levels in penguins on entry and during the rehabilitation process.

MATERIALS AND METHODS

Sample collection and processing

Natural immunity of African penguins to avian malaria on entry to the SANCCOB facility and for up to 3 weeks after submission was assessed for the period from October 2001 to January 2003.

Blood samples from a total of 30 penguins present in the SANCCOB rehabilitation facility (Tableview, Cape Town) were obtained on a weekly basis. Sample collection was done in a manner so that it would firstly bring about minimal additional work and was therefore collected along with routine blood samples taken weekly for malaria blood smears. This single blood collection was purposely planned in order to not add additional stress to the penguin during the rehabilitation process. Secondly, it was important not to incur additional cost to the rehabilitation procedures at SANCCOB. This meant that the release of apparently healthy birds was not delayed just because they were in this trial, resulting in a difference in the number of data points collected

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for each bird. Released birds were, however, replaced so as to always have a total of 30 penguins, unless fewer birds were actually being rehabilitated, at any given time.

Blood was collected as described by Graczyk *et al.* (1994c), except that only a single 50 µl blood volume, as measured by calibrated heparinized capillary tubes, was deposited on filter paper (Immunovet Services) and air dried. Prior to ELISA testing, blood was eluted overnight at 4°C from each of the filter papers with 333.33 µl of Millipore water containing 0.4% Tween 20 (Graczyk *et al.*, 1993).

A negative serum sample was obtained from a penguin chick that was artificially hatched in isolation. Sera with low, medium and high anti-*Plasmodium* antibody levels were obtained from penguins being rehabilitated at SANCCOB.

The rabbits used for immunization purposes were of mixed stock and obtained from the breeding colony of the Department of Biochemistry, University of Stellenbosch, South Africa.

ELISA Capture Antigen

A malaria peptide, with a sequence corresponding to the repeat region of the CS-protein of *P. falciparum*, was synthesized for use as capture antigen in the malaria antibody ELISA (Ballou *et al.*, 1985). The 16 amino acid peptide, with the sequence $(NANP)_4$, was synthesized by the BIOPEP Peptide Synthesis Laboratory at the Biochemistry Department, University of Stellenbosch. Prior to use, the peptide was conjugated to BSA, which consisted of three reaction steps, i.e. introduction of maleimide groups into BSA; introduction of a sulphydryl group into the malaria peptide and conjugation of the malaria peptides to the activated BSA (Klotz and Heiney, 1959; Rector *et al.*, 1978; Yoshitake *et al.*, 1982; Partis *et al.*, 1983; Hashida *et al.*, 1984). By using different ratios of malaria peptide to maleimide derivitized BSA (1:1, 3:1 and 9:1) three conjugates of different malaria peptide to BSA ratios were generated. These conjugates were referred to as 1:1, 3:1 and 9:1 malaria peptide to BSA conjugates.

Preparation of rabbit anti-penguin Ig antibodies

Blood was drawn from penguins housed at the SANCCOB rehabilitation facility and serum isolated. Penguin immunoglobulin (Ig) was purified from this serum as follows. Briefly, penguin serum was diluted (1:2) with phosphate buffered saline (PBS,

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0.15 M, pH 7.2) and Ig precipitated by the addition of an equal volume of saturated ammonium sulphate solution at 4°C. After centrifugation at 27 200 x g for 20 min the supernatant was removed and the precipitate dissolved in PBS. This precipitation step was then repeated. Finally the precipitate was dissolved in an amount of PBS equivalent to the original serum volume and dialyzed overnight at 4°C against PBS, changing buffer once during dialysis. The Ig concentration in the final dialysate was determined by absorption (280 nm) using BSA as standard. This isolated penguin Ig was subsequently adsorbed to acid-treated naked bacteria as described and used for the immunization of rabbits (Bellstedt *et al.*, 1987).

A rabbit was immunized intravenously into the marginal ear vein with the penguin Ig-NB complexes (200 μg NB /immunization) on days 1, 3, 7, 14, 21, 28, 32 and 35. Blood was collected on days 1, 14, 28 and 42, after which the rabbit anti-penguin Ig titres were determined using the ELISA described hereafter.

ELISA for rabbit anti-penguin Ig antibodies

Microtitre plates (96 well, Nunc Maxisorp with certificate, Denmark) were coated with penguin Ig (2 μg/ml, 100 μl/well) in carbonate buffer (50 mM, pH 9.6) overnight at 4°C. The coating solution was decanted and the plate blocked with Casein buffer (0.5% casein, 0.15 M NaCl, 0.01 M Tris-HCl, 0.02% thiomersal, pH 7.6, 200 μl/well) for 1 h at 37°C. The rabbit sera were pre-diluted 1/20 with Casein buffer containing 0.1% Tween 20 (Casein-Tween). Serial dilutions of the sera to be tested were made in Casein-Tween using a total volume of 100 μl/well and incubated for 1 h at 37°C. Sheep anti-rabbit antibody (Sigma), diluted 1/400 in Casein-Tween was added next (100 μl/well) and incubated for 1 h at 37°C. Rabbit peroxidase anti-peroxidase complex (PAP, Sigma), diluted 1/10 000 in Casein-Tween, was added (100 μl/well) and incubated for 1 h at 37°C. Finally, substrate solution was added (0.05% 2,2'-Azino-bis[3-ethylbenzothiazoline-6-sulfonic acid] (ABTS), 0.015% H_2O_2 in 0.1 M citrate buffer, pH 5, 100 μl/well) and the absorbance measured at 405 nm after 30 min at 37°C on a Labsystems Multiskan MS spectrophotometer. Between all steps, except between the coating and blocking step, the plates were washed three times with PBS (0.15 M, pH 7.2) containing 0.1% Tween 20 (PBS-Tween) (200 μl/well).

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The anti-penguin Ig antiserum titres were expressed as the dilution of rabbit anti-penguin antiserum giving an absorbance of 0.1 in the above ELISA (Bellstedt *et al.*, 1987).

Biotinylation of rabbit anti-penguin antibodies

The Ig fraction of the rabbit anti-penguin Ig antiserum was isolated using ammonium sulphate precipitation as previously described for penguin Ig, except that after dissolving the final precipitation, it was dialyzed overnight against several changes of Carbonate buffer (0.1 M, pH 8.3) at 4°C. The Ig concentration in the final dialysate was determined by absorption (280 nm) using BSA as standard. Carbonate buffer was then added in order to obtain an Ig concentration of 5 mg/ml. A solution (2 mg/ml) of biotinamidocaproate N-hydroxysuccinimide ester (Biotin, Sigma) in N,N-dimethylformamide (DMF) was prepared and added to the above mentioned Ig solution while sirring slowly for 2 h at RT. The Biotin-penguin Ig conjugate was dialyzed overnight against several changes of PBS (0.15 M, pH 7.2) at 4°C. Glycerol was added to the final dialysate in a 1:1 ratio and stored at -20°C.

Penguin anti-*Plasmodium* antibody ELISA

Microtitre plates (96 well, Nunc, MediSorp, Denmark) were coated with 0.5 μg/ml (100 μl/well) of the malaria peptide-BSA conjugate (a 3:1 ratio conjugate was determined to be optimal) in Carbonate buffer (50 mM, pH 9.6) overnight at 4°C. The coating solution was decanted and the plate blocked with Casein buffer (0.5% casein, 0.15 M NaCl, 0.01 M Tris-HCl, 0.02% thiomersal, pH 7.6, 200 μl/well) for 1 h at 37°C. Blood eluted from filterpaper was diluted 1/50 and serum samples 1/200 in Casein-Tween. Samples were added to wells (100 μl/well) in triplicate and incubated for 3h at 37°C. This was followed by the addition of biotinylated rabbit anti-penguin antibody, diluted 1/100 in Casein-Tween (100 μl/well) and incubated for 1 h at 37°C. Avidin-horseradish-peroxidase conjugate (Zymed) diluted 1/100 in Casein-Tween was then added (100 μl/well) and incubated for 1 h at 37°C. Finally the substrate (ABTS, 0.015% H_2O_2 in 0.1 M citrate buffer, pH 5, 100 μl/well) was added and the absorbance measured at 405 nm after 30 min at 37°C on a Labsystems Multiskan MS spectrophotometer. Between all steps the plates were washed three times (200 μl/well) with PBS-Tween, except between the coating and blocking steps.

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Inter-assay variations were monitored by the inclusion of standard penguin sera with negative, low, medium and high anti-*Plasmodium* antibody levels.

Data analysis

For analysis purposes the ELISA data obtained over the 16-month period was grouped together into two-month periods as shown in Figures 2 and 3. Penguins were continuously being admitted and to simplify analysis, the date of entry was taken as week 1 and subsequent weeks of sampling within a specific two-month period as weeks 2, 3 and 4 respectively (except for the Dec 2002 – Jan 2003 period where sampling was only done for 3 weeks). Statistical analysis of the data was performed using the General Linear Models (GLM) procedure in the Statistical Analysis System (SAS) Enterprise Guide (version 1.3.0.161). Most penguins seldom stayed at the facility for longer than 3 – 4 weeks during rehabilitation. As a result of too many missing values from week 4 onwards, only the first three data points were used for statistical analysis. Only average ELISA titre values were plotted for each week in each respective two-month period.

RESULTS

The immune response of the rabbit to penguin Ig is graphically depicted as antibody titre plotted against time (Figure 1). This rabbit anti-penguin Ig antiserum was subsequently used for the development of an ELISA for the detection of malaria antibodies in penguins.

The synthesized *Plasmodium* peptide was conjugated to BSA in a 1:1, 3:1 and 9:1 ratio for use as coating antigen in the anti-*Plasmodium* ELISA. The different peptide-BSA ratios were tested at different coating concentrations and a 3:1 ratio at a concentration of 0.5 μg/ml was found to be optimal and was used in subsequent anti-*Plasmodium* antibody determinations in the ELISA.

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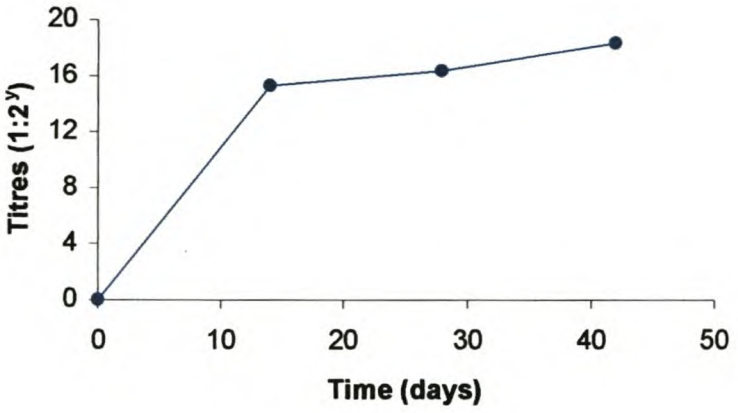

Figure 1: The anti-penguin Ig immune response of a rabbit immunized with penguin Ig adsorbed to naked bacteria, as determined by ELISA. The exponential of the reciprocal titre is indicated on the Y-axis.

To determine whether the ability of penguins to produce an anti-*Plasmodium* antibody response influenced their survival during rehabilitation, the anti-*Plasmodium* antibody levels of the penguins that were released and those that died during rehabilitation were compared (Figure 2A, B and C). Although statistical analysis of this data indicated that the penguins were responding to *Plasmodium* infections by producing an immune response (P-values showing significant time responses on individual graphs), the difference in response between the penguins that were released and those that died was not statistically significant.

The effect of oiling on the immune response of birds to *Plasmodium* infections is illustrated in Figure 3A, B and C. Statistical analysis indicated no significant difference in the anti-*Plasmodium* immune response between penguins that were oiled and those that were not oiled. There was, however, a significant difference ($P = 0.0136$) in response between the oiled and the non-oiled group in the period December 2001 to January 2002. The significance of this result is questionable as only 2 out of 7 penguins created this difference in the oiled group compared to 41 penguins in the non-oiled group. An average ELISA titre of 2.124 for these two penguins compared to an average of 0.179 for the other five penguins placed doubt on this analysis.

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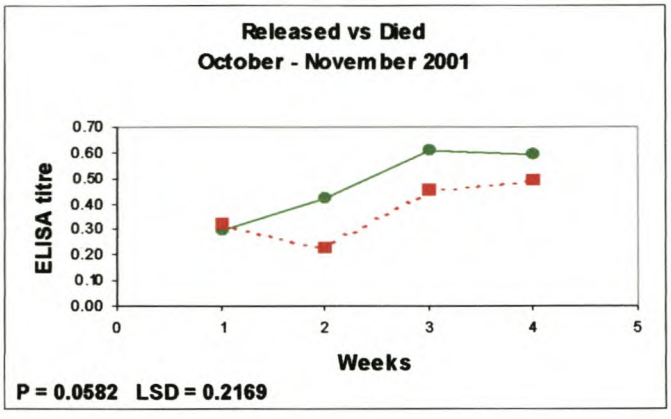
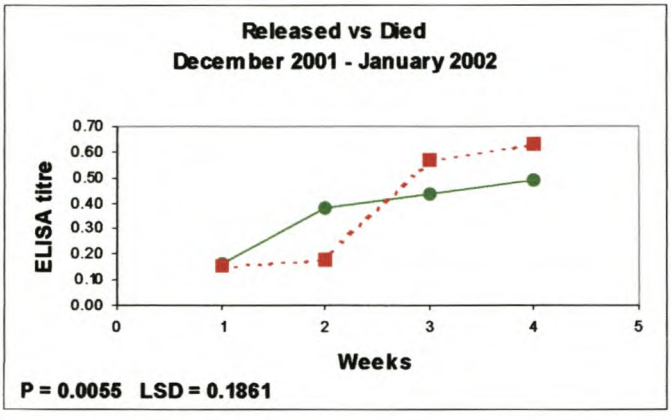
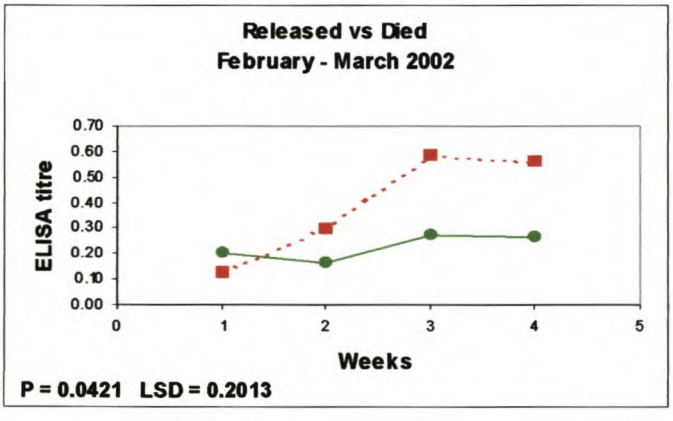


Oct – Nov 2001		
Bloodsmear +		Malaria deaths/ Total bird number
Released	19/36	
Died	12/16	12/52
Total	31/52	



Dec 2001 – Jan 2002		
Bloodsmear +		Malaria deaths/ Total bird number
Released	12/25	
Died	22/23	22/48
Total	34/48	



Feb – Mrt 2002		
Bloodsmear +		Malaria deaths/ Total bird number
Released	3/6	
Died	2/4	2/10
Total	5/10	

Figure 2A: Anti-*Plasmodium* immune response of penguins that were released (•) versus those that died (■) during rehabilitation for the period October 2001 to March 2002. No statistically significant treatment time interaction was found within any of the two-month periods, which means that there was no difference between the immune response of the penguins that were released and those that died. The given P values indicate the statistical significance of the time response. Malaria bloodsmear data obtained from penguins for each of the respective two-month periods are shown opposite each immune response graph along with the number of penguins that died due to malaria.

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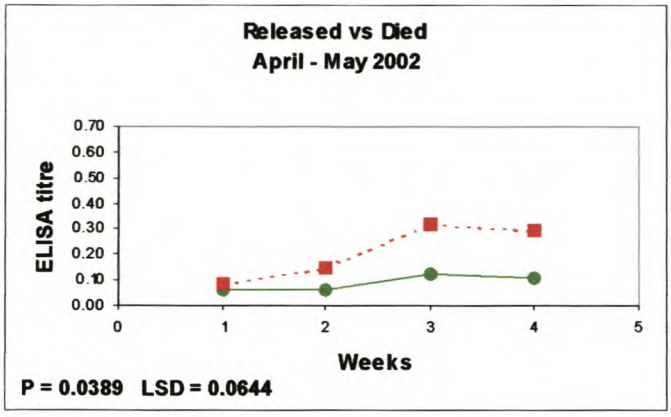
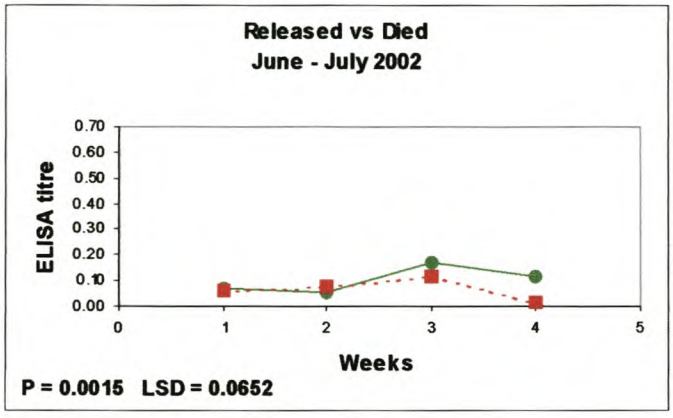
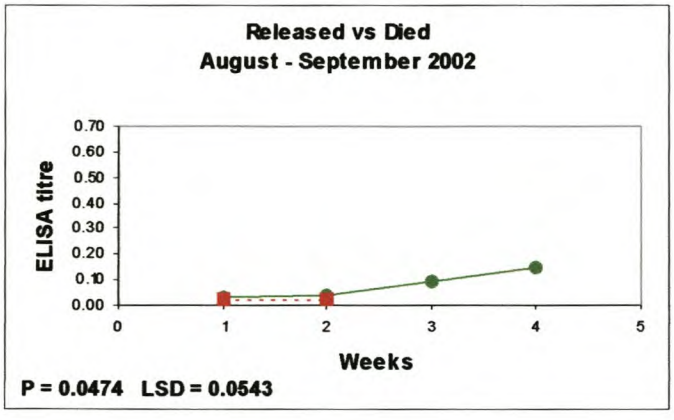


Apr – May 2002		
Bloodsmear +		**Malaria deaths/ Total bird number**
Released	7/59	
Died	4/7	4/66
Total	11/66	



Jun – Jul 2002		
Bloodsmear +		**Malaria deaths/ Total bird number**
Released	4/46	
Died	1/5	1/51
Total	5/51	



Aug – Sept 2002		
Bloodsmear +		**Malaria deaths/ Total bird number**
Released	9/59	
Died	0/2	0/61
Total	9/61	

Figure 2B: Anti-*Plasmodium* immune response of penguins that were released (●) versus those that died (■) during rehabilitation for the period April 2002 to September 2002. No statistically significant treatment time interaction was found within any of the two-month periods, which means that there was no difference between the immune response of the penguins that were released and those that died. The given P values indicate the statistical significance of the time response. Malaria bloodsmear data obtained from penguins for each of the respective two-month periods are shown opposite each immune response graph along with the number of penguins that died due to malaria.

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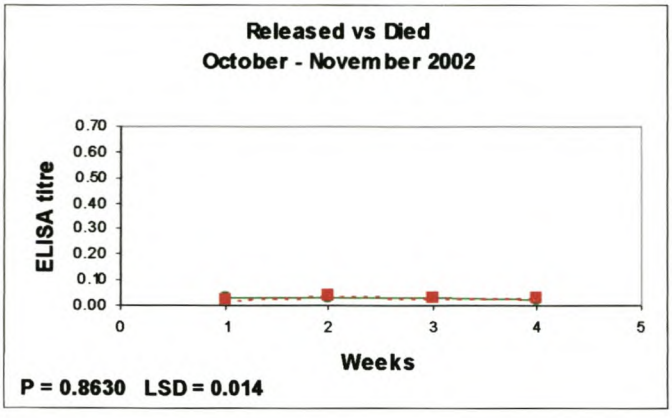
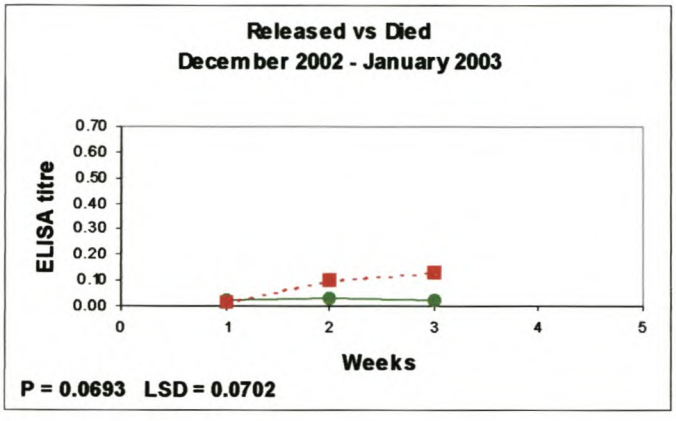


Okt – Nov 2002		
Bloodsmear +		Malaria deaths/ Tota bird numberl
Released	5/35	
Died	4/7	4/42
Total	9/42	



Dec 2002 – Jan 2003		
Bloodsmear +		Malaria deaths/ Total bird number
Released	9/22	
Died	3/10	3/32
Total	12/32	

Figure 2C: Anti-*Plasmodium* immune response of penguins that were released (●) versus those that died (■) during rehabilitation for the period October 2002 to January 2003. No statistically significant treatment time interaction was found within any of the two-month periods, which means that there was no difference between the immune response of the penguins that were released and those that died. The given P values indicate the statistical significance of the time response. Malaria bloodsmear data obtained from penguins for each of the respective two-month periods are shown opposite each immune response graph along with the number of penguins that died due to malaria.

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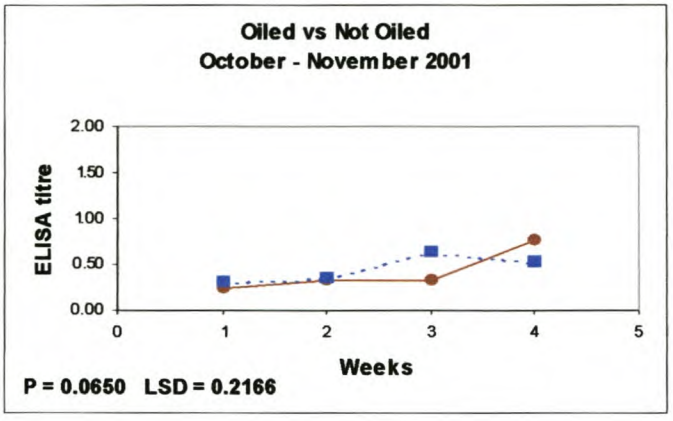
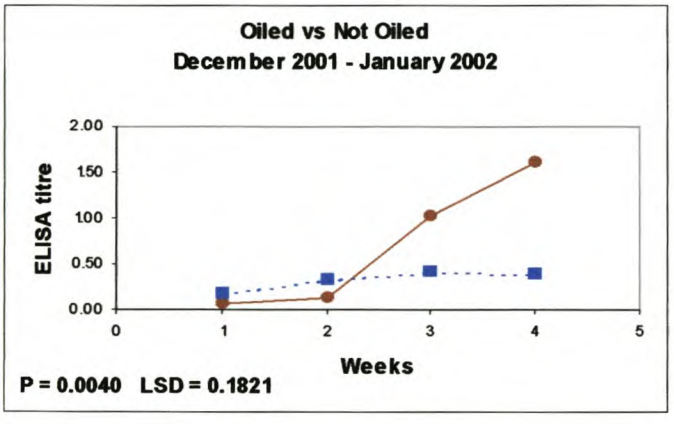
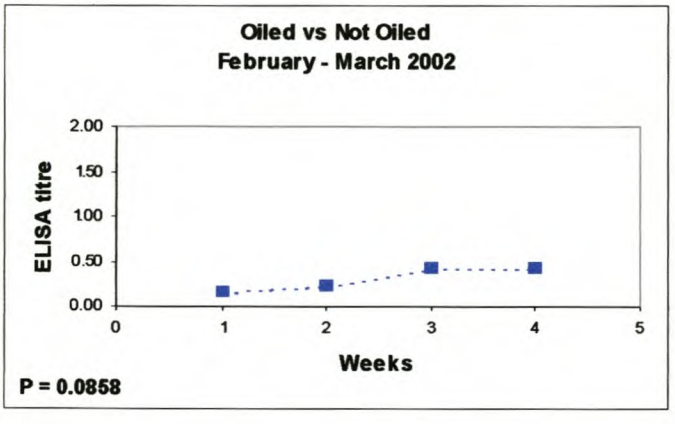


Oct – Nov 2001		
Bloodsmear +		Malaria deaths/ Total bird number
Oiled	9/20	2/20
Non-Oiled	22/32	10/32
Total	31/52	



Dec 2001 – Jan 2002		
Bloodsmear +		Malaria deaths/ Total bird number
Oiled	5/7	3/7
Non-Oiled	29/41	19/41
Total	34/48	



Feb – Mar 2002		
Bloodsmear +		Malaria deaths/ Total bird number
Oiled	0	0
Non-Oiled	5/10	2/10
Total	5/10	

Figure 3A: Anti-*Plasmodium* immune response of penguins that were oiled (•) versus those that were not oiled (■) on admission to SANCCOB for the period October 2001 to March 2002. A statistically significant treatment time interaction (P = 0.0136) was only obtained for the period December 2001 to January 2002. No LSD value is given for the February to March 2002 group as there were no oiled birds in this period. The given P values indicate the statistical significance of the time response. Malaria bloodsmear data obtained from penguins for each of the respective two-month periods are shown opposite each immune response graph along with the number of penguins that died due to malaria in each of the oiled and non-oiled groups.

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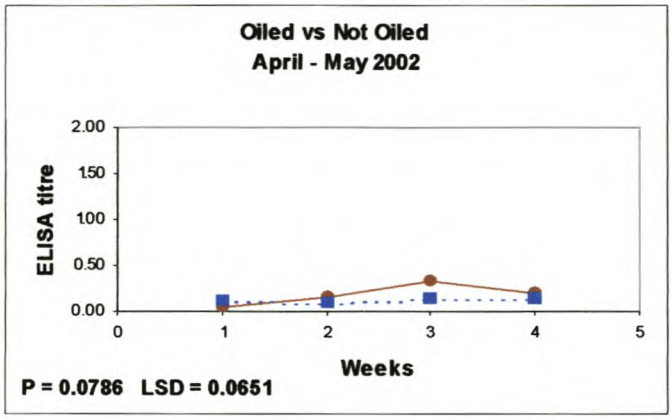
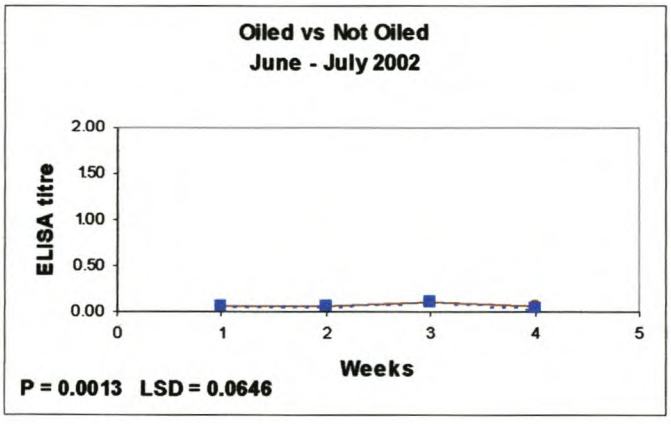
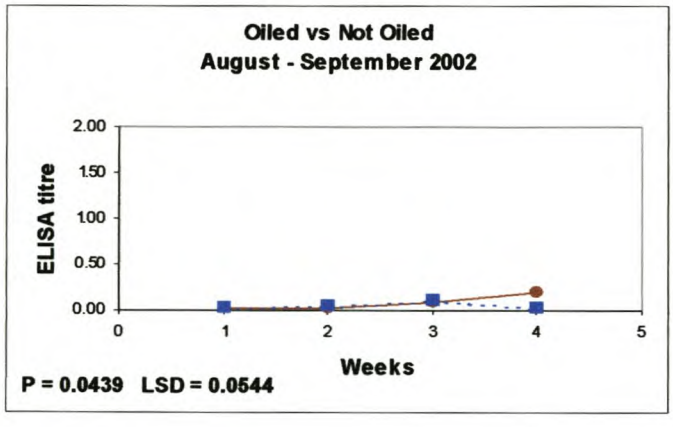


Apr – May 2002		
Bloodsmear +		Malaria deaths/ Total bird number
Oiled	5/41	3/41
Non-Oiled	6/25	1/25
Total	11/66	



Jun – Jul 2002		
Bloodsmear +		Malaria deaths/ Total bird number
Oiled	3/43	0/43
Non-Oiled	2/8	1/8
Total	5/51	



Aug – Sept 2002		
Bloodsmear +		Malaria deaths/ Total bird number
Oiled	4/46	0/46
Non-Oiled	5/15	0/15
Total	9/61	

Figure 3B: Anti-*Plasmodium* immune response of penguins that were oiled (●) versus those that were not oiled (■) on admission to SANCCOB for the period April 2002 to September 2002. No statistically significant treatment time interaction was found within any of the two-month periods, which means that there was no difference between the immune response of the penguins that were oiled and those that were not oiled. The given P values indicate the statistical significance of the time response. Malaria bloodsmear data obtained from penguins for each of the respective two-month periods are shown opposite each immune response graph along with the number of penguins that died due to malaria in each of the oiled and non-oiled groups.

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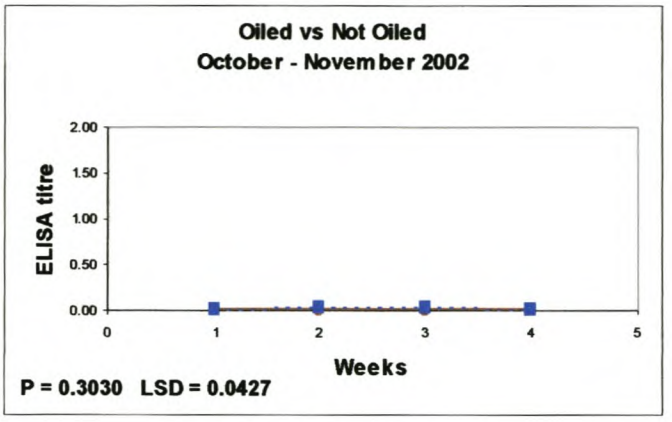
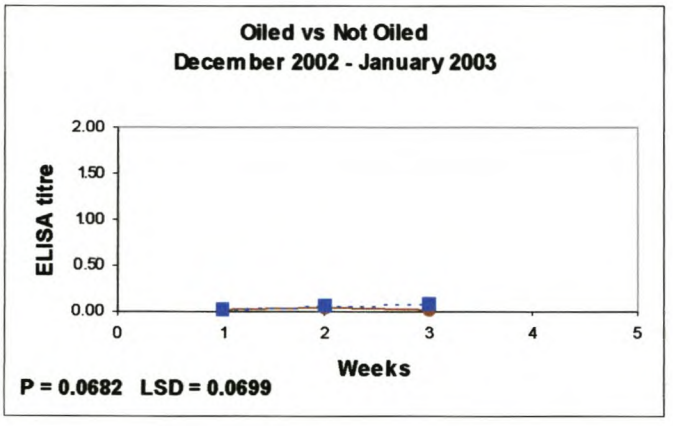


Okt – Nov 2002		
Bloodsmear +		Malaria deaths/ Tota bird numberl
Oiled	2/19	0/19
Non-Oiled	7/23	4/23
Total	9/42	



Dec 2002 – Jan 2003		
Bloodsmear +		Malaria deaths/ Total bird number
Oiled	3/4	1/4
Non-Oiled	9/28	2/28
Total	12/32	

Figure 3C: Anti-*Plasmodium* immune response of penguins that were oiled (•) versus those that were not oiled (■) on admission to SANCCOB for the period October 2002 to January 2003. No statistically significant treatment time interaction was found within any of the two-month periods, which means that there was no difference between the immune response of the penguins that were oiled and those that were not oiled. The given P values indicate the statistical significance of the time response. Malaria bloodsmear data obtained from penguins for each of the respective two-month periods are shown opposite each immune response graph along with the number of penguins that died due to malaria in each of the oiled and non-oiled groups.

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In all the above data, higher antibody responses were observed during spring and summer (October – March) than in autumn and winter (April – September). This correlates with the fact that there is an increase in the number of birds that are diagnosed malaria positive by bloodsmear and the number of birds that die due to malaria in the summer months. The number of malaria bloodsmear positive birds drop with the start of autumn (April) with resulting lower mortalities due to malaria.

From October 2001, there was an increase in antibody levels within the first two weeks after entering the facility and this pattern continued until May 2002. This suggests that birds are either responding to new malaria infections, or that the antibody response seen is due to parasite recrudescence as a possible result of stress during rehabilitation. The high anti-*Plasmodium* antibody levels seen during the spring and summer of 2001/2002 were not repeated in the spring and early summer of 2002/2003.

DISCUSSION

The survival of penguins with *Plasmodium* infections is influenced by their ability to produce an anti-*Plasmodium* immune response. The speed at which this immune response occurs is vital to the control of infection. If the response is too slow, the bird will succumb (Seed and Manwell, 1977). Given the presence of premunition, birds would normally quickly counterbalance and thus control the infection with an intensified immune response (Huff, 1963; Seed and Manwell, 1977). In this study, both penguins that were released and those that died produced an anti-*Plasmodium* immune response, but the difference in this immune response was not statistically significant. The mortality results can, however, be viewed as being biased due to the fact that all penguins diagnosed to be malaria positive by blood smear were treated against malaria thereby preventing subsequent death. The number of penguins that were diagnosed malaria positive by bloodsmear was higher during the warmer spring and summer months than during the colder autumn and winter months. This coincided with a higher anti-*Plasmodium* immune response in the warmer spring and summer months, which indicates that the penguin anti-*Plasmodium* ELISA is in fact measuring anti-*Plasmodium* antibody levels.

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Oiling is currently one of the major human threats to the conservation of the African penguin. Oiling causes penguins to loose their natural insulative and waterproofing properties, which can lead to hypothermia, dehydration, starvation and eventually death. In an attempt to remove the oil by preening themselves, penguins ingest oil that can lead to internal organ damage as well as anaemia and a depression of immune function (Briggs *et al.*, 1996; Nel *et al.*, 2003). An altered immune function in oiled birds could make them more susceptible to malaria infections during rehabilitation. In this study, oiling was found not to influence the ability of penguins to produce an anti-*Plasmodium* immune response. Penguins become oiled whilst spending time in the water and only strong and healthy penguins go out to sea. Given that penguins are brought to SANCCOB as soon as possible after oiling, oiled birds that arrive at SANCCOB are strong and this may therefore explain why their anti-*Plasmodium* immune responses are not different from that of non-oiled penguins. In addition to this, the number of oiled penguins that was diagnosed with malaria by bloodsmear was consistently lower than the number of non-oiled malaria bloodsmear positive penguins (except for Dec 2002 – Jan 2003). This once again indicated that oiling had no effect on the prevalence of malaria in penguins that are being rehabilitated.

It is assumed that once penguins become infected, they remain infected for life (Cranfield *et al.*, 1990). If penguins survive their first infection with avian malaria, their immune system appears to be capable of reducing the number of parasites to subpatent levels. This results in a low-grade infection due to survival of latent parasites and is referred to as premunition. Recrudescence of parasites can subsequently be induced in birds with premunition by stress factors such as nutritional-, environmental- or migration stress (Griner, 1974). The rehabilitation process also puts the penguins under stress, which could lead to recrudescence in penguins with existing infections. However, if recrudescence were the main cause of antibody responses seen in penguins being rehabilitated at SANCCOB, then the same increase in level of immunity after entry would be seen right throughout the year, irrespective of environmental conditions. The results obtained in this study clearly show this not to be the case. Antibody levels to avian malaria were much higher in the spring and summer period of 2001/2002 than those seen during the spring and summer period of 2002/2003 which can be explained by differences in

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weather patterns from one year to the next. Antibody levels declined during autumn (April – May 2002) and stayed low during the whole winter. Cooler temperatures have a negative influence on the distribution of mosquito vectors with a subsequent decrease in malaria infections. The absence of an antibody response in penguins during the winter months further illustrates the fact that avian malaria infections are seasonal due to infections being dependent on the presence of a suitable mosquito vector. The immune responses seen in the warmer months can therefore in all likelihood be ascribed to birds being infected by mosquitoes and not parasite recrudescence.

Results therefore indicate penguins to become infected with avian malaria during their rehabilitation at the SANCCOB facility. SANCCOB is an open-air facility and is adjacent to a large shallow freshwater lake with abundant bird life, thereby bringing susceptible penguins in close proximity to both avian malaria vectors and potential avian malaria reservoir hosts. Unless conditions at the facility can be modified to either minimize or completely eliminate exposure to mosquito vectors, the repositioning of the facility should be considered. Future investigations will have to be undertaken to determine the prevalence of avian malaria in wild penguin colonies in order to determine the role of SANCCOB in introducing avian malaria into wild penguin populations.

Currently the status of the African penguin is listed as 'Vulnerable' in both the South African Red Data Book for birds and the World conservation Union Red List of Threatened Species™. An assessment of the impact of avian malaria on African penguin populations is thus needed for both effective management of this disease during rehabilitation as well as in improving future conservation efforts.

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CHAPTER 8:

Summary and future perspectives

In Chapter 3 the transfer of maternal antibodies to ostrich egg yolk was assessed and maternal antibodies were found to be transferred to the egg yolk. Vaccination of ostrich hens would thus ensure that the progeny is provided with anti-NDV antibodies after hatch. In the second section of this chapter, ostrich IgA was isolated, secondary anti-ostrich IgA antibodies produced and an anti-NDV IgA ELISA developed. This ELISA was used to show that immunization of ostrich chicks with live La Sota vaccine induced mucosal immunity. The availability of the ostrich IgA secondary antibodies produced in this study will facilitate future evaluation of mucosal immunity to NDV and subsequent development of optimal vaccination schedules in ostrich chicks. This will have to include different dosages and different NDV vaccine strains in the presence or absence of maternal immunity. Additionally, the immune responses should be followed over longer periods in order to document the duration and period of maximal IgA production. The ostrich IgA secondary antibodies can also be used effectively for the characterization and evaluation of mucosal immunity against any ostrich disease affecting the respiratory tract such as mycoplasma infections.

In Chapter 5 the identification of three novel mycoplasmas from ostriches in the Klein Karoo, Great Karoo and Garden Route areas of South Africa was described. Specific primers for the detection of each of these mycoplasmas were developed for diagnostic purposes. These three mycoplasmas where phylogenetically compared to existing avian mycoplasmas and were found to fall into two distinct phylogenetic groupings. This knowledge will now be used in the future development of vaccines. The phylogeny indicates why vaccination attempts with the current poultry mycoplasma vaccine against *M. gallisepticum* were ineffective. The phylogeny also indicates that the use of *M. synoviae* vaccines may prove to be successful due to the close relatedness between *M. synoviae* and Ms03 and even Ms02. No vaccines are currently available against close relatives of Ms01 indicating that a specific vaccine for this mycoplasma will have to be developed. In future research we plan to determine if Ms01 possesses a cytadhesin protein similar to that of the Gap A gene in *M. gallisepticum* as a possible candidate for vaccine development.

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In Chapter 7 the immunity of African penguins at the SANCCOB rehabilitation facility to avian malaria was determined. Results indicated that the increase in immune response was the result of new infections and not recrudescence. The assessment of this immunity has already been extended over a further calendar year in combination with infection data that will be obtained using a specific PCR for the detection of *Plasmodium* infections in African Penguins. This expanded data set together with the data presented in this thesis will be used to decide whether African penguins are infected with avian malaria prior to their arrival or during rehabilitation at the SANCCOB facility. Should the latter be the case, repositioning of the facility should be considered in order to reduce mortalities during rehabilitation. This would also imply that rehabilitation at SANCCOB is responsible for the introduction of avian malaria into wild populations, which may play a negative role in nature. These studies will in future also be expanded to include penguins from several land and island colonies to assess the role avian malaria may play in the survival of the African penguin in the wild.

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Appendix

16S rRNA GENE SEQUENCES OF Ms01, Ms02, Ms03, *M. SYNOVIAE* AND *M. GALLINACEUM*

```
                                                                                                  60
Ms01          ----CTGGCT GTGTGCCTAA TACATGCATG TCGAGCGGGG TTTTTTAA-A CCT--AGCGG
Ms02          ----...... .......... .......... ........A. ..C..CGG-. A..T-.....
Ms03          ----...... .......... .......... ........A. ..C..CGG-. A..T-.....
Synoviae      ----...... .......... .......... ........AA ...AGC..T. AA.TT.....
Galnaceu      ----...... .......... .....R.... ........A. ..C..CGG-. A..T-.....

                                                                                                 120
Ms01          CGAATGGGTG AGTAACACGT GCTTAATCTA CCTTTTAGAT TGGAATACCT GATGGAAACA
Ms02          .......... .......... A.....CG.G ..C.C..... G......A.G AT.A....TG
Ms03          .......... .......... A.....CG.G .........C .......A.A CTGA....TT
Synoviae      .......... .......... A.....CG.. .........C .......A.G .TGA....TT
Galnaceu      .K........ .......... A.....CG.G ..CCC.TR.. ...G...A.G CTGA....TT

                                                                                                 180
Ms01          TTAGTTAATG CCGGATACGC AT-GGAATCG CATGATTCCG TTGTGAAAGG GGCCTC-AAA
Ms02          A.C.C..... ...A....TT ..TA.CT... .....AGTTA A.A.A..... A..G.TT---
Ms03          AGT.C..... ........TT ..ACA..... .......TGT A.A......A A.....-...
Synoviae      A.C.C..... .......TAT .AAAA..... .......TTT ..AA.....A A..G.TT---
Galnaceu      AGC.C....A ........TT .AATATC... .....GGTAT ..A.A..... A....TT...

                                                                                                 240
Ms01          GCCCCACTAA GAGATGAGGG TGCGGAACAT TAGTTAGTTG GTAGGGTAAT GGCCTACCAA
Ms02          ..TT.G...G AG...CG... ....T..... .......... ..GA...... ...TC.....
Ms03          ..TT...... A....CG... ....T..... ...C...... ..GA.....C ....C.....
Synoviae      ..TT...... .....CG... .......... ...CAT.... .AT....... ..........
Galnaceu      ..TT...A.G .G...CG... ....T..... ...C...... ..GA...... ...TC.....

                                                                                                 300
Ms01          GACTATGATG TTTAGCCGGG TCGAGAGACT GAACGGCCAC ATTGGGACTG AGATACGGCC
Ms02          ...G...... ......G... .T........ ..T.C..... .C........ ..........
Ms03          .G........ ......G... .T.......C ....C..... .C........ ..........
Synoviae      .G.A..T... ......G... .T........ ....C..... .C.......A GAGAT.....
Galnaceu      .G.G...... ......G... .T........ ..T.C..... .C........ ..........

                                                                                                 360
Ms01          CAAACTCCTA CGGGAGGCAG CAGTAGGGAA TATTCCACAA TGAGCGAAAG CTTGATGGAG
Ms02          ..G....... .......... .......... .T........ ..G....... .C........
Ms03          ..G....... .......... .......... .T........ ..G....... .C........
Synoviae      ..G....... .......... ...AT..... .T........ ..G....... ..........
Galnaceu      ..G....... .......... .......... .T........ ..G....... .C........
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                                                                                                  420
Ms01        CGACACAGCG TGCACGATGA AGGTCTTCGG ATTGTAAAGT GCTGTTATAA GGGAAGAACA
Ms02        .......... ...G..... ...C..... G........C .......... ........A.
Ms03        .......... ....G..A.. C..C..... G.......C. ........TT A...T...A.
Synoviae    .A........ ..T.G..... ...C..... G.......C. A........T ........A.
Galnaceu    .......... ....G..... ...C-.... G.......C. .......... ........A.

                                                                                                  480
Ms01        TTCAAAAGAG GAAAT-GCTT TTGAACTGAC GGTACCTTAT CAGAAAGCGA TGGCTAACTA
Ms02        AG..GT.... .....-...A ...CCT.... ........G. ........A. C.........
Ms03        AA.CTTGC.. .....G..AA -G.CCT.... A....TAA.. ........A. C.........
Synoviae    ACT.GT.T.. .....-.A.A ..AGCT.... ......A.G. ........A. C.........
Galnaceu    AGTCG.G... .....-..C. .GACCT.... .......... ........A. C.........

                                                                                                  540
Ms01        TGTGCCAGCA GCCGCGGTAA TACATAGGTC GCAAGCGTTA TCCGGAATTA TTGGGCGTAA
Ms02        .......... .......... .........T .......... .......... ..........
Ms03        .......... .......... .........T .......... .......... ..........
Synoviae    .......... .......... .........T .......... .......... ..........
Galnaceu    .......... .......... .........T .......... .......... ..........

                                                                                                  600
Ms01        AGCGTTCGTA GGCTGTTTGT TAAGTCTGGA GTCAAATCCC GGGGCTCAAC CCCGGCTCGC
Ms02        .....CT... ..T....... .........T ..G...A.TT ..A....... T..AAA.T..
Ms03        .....CT... ..T...G... .........C ......A.TT .......... ...AAA....
Synoviae    .....CT... ..T....... .........T ..T...A.TT ..A....... T..AAA.T..
Galnaceu    .....CT... ..T....... .........T ..G...A.TT .......... ...AAA.T..

                                                                                                  660
Ms01        TTTGGATACT GGCAAACTAG AGTTGGATAG AGGTAAGCGG AATTCCATGT GAAGCGGTGA
Ms02        A......... .......... ....ATG... .......... ......T... .........G
Ms03        G......... ....CG.... .A...T.... ....T.A... ......T... ..........
Synoviae    A......... ....G..... .A...TT... ....T..... ......T... .........G
Galnaceu    A......... .......... .A...TG... ....T..... ......T... .........G

                                                                                                  720
Ms01        AATGCGTAGA TATATGGAAG AACACCAAA- -GGCGAAGGC AGCTTACTGG GTCTATACTG
Ms02        .......... ....A..... ........CC T......... .......... ACA.......
Ms03        .......... ....A..... ........CA T......... ..T.A..... ..AC...T..
Synoviae    .......... ....A..... ........CA T......... ....A..... .AAC...T..
Galnaceu    .......... ....A..... ....T...CA T......... ....A..... ACA....T..

                                                                                                  780
Ms01        ACGCTGAGGG ACGAAAGCGT GGGGAGCAAA CAGGATTAGA TACCCTGGTA GTCCACGCTG
Ms02        ..A.....A. .......... .......... .......... .......... ........C.
Ms03        ..A.....A. .......... .......... .......... .......... ..........
Synoviae    ..A.....A. .......... .......... .......... .......... ..........
Galnaceu    ..A.....A. .......... .......... .......... .......... ........C.

                                                                                                  840
Ms01        TAAACGATGA TCATTAGTCG GTGGGGAACT CA-CTGACGC AGCTAACGCA TTAAATGATC
Ms02        .......... .G.....CT. A......G.. ..T.-.G... .......... ......C...
Ms03        .......... .G.....CT. A.A..A.-.. -.T.-.G... .......... ......C...
Synoviae    .......... .G.C....T. A.A.AA.-.- ..T.-..... .CG....... ....G.C...
Galnaceu    .......... .G.....CT. A......... ..T.-.G... .CG....... ......C...
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                                                                                                  900
Ms01        CGCCTGAGTA GTATGCTCGC AAGAGTGAAA CTTAAAGGAA TTGACGGGGA CCCGCACAAG
Ms02        .......... .......... .......... .......... .......... T.........
Ms03        .......... .......... .......... .......... .......... T.........
Synoviae    .......... .......... .......... .......... .......... T.........
Galnaceu    .......... .......... .......... .......... .......... T.........

                                                                                                  960
Ms01        CGGTGGAGCA TGTGGTTTAA TTTGAAGATA CACGGAGAAC CTTACCCACT CTTGACATCT
Ms02        .......... .......... .......... .G..T..... .......... ..........
Ms03        .......... .......... .......... .G..T..... .......... ..........
Synoviae    .......... .......... .......... .G..T..... .......... ..........
Galnaceu    .......... .......... .......... .G..T..... .......... ..........

                                                                                                 1020
Ms01        TC-TGCAAAG CTATAGAGAT ATAGTGGAGG TTAACAGAAT GACAGATGGT GCATGGTTGT
Ms02        ..C-...... .......... .......... C....G.... .......... ..........
Ms03        ..-....... .......... .......... .C........ .......... ..........
Synoviae    ..T....... .......... .......... .......... .......... ..........
Galnaceu    ..-....... .......... .......... .......... .......... ..........

                                                                                                 1080
Ms01        CGTCAGCTCG TGTCGTGAGA TGTTTGGTCA AGTCCTGCAA CGAGCGCAAC CCCTATCTTT
Ms02        .......... .......... ....C...T. .......... .......... ..T.G..C..
Ms03        .......... .......... ....C...T. .......... .......... ..T...TC..
Synoviae    .......... .......... ....C...T. .......... .......... ..T.G..C..
Galnaceu    .......... .......... ....C...T. .......... .......... ..T.G..C..

                                                                                                 1140
Ms01        AGTTACTAAC GAGTTAAGTC GAGGACTCTA GAGATACTGC CTGGGTAACC GGGAGGAAGG
Ms02        .T...GATGA T.T--...GA ..-------- ------.... .C.......T ..........
Ms03        .....AATCT TCT--.G.GA ..-------- ------.... .C........ ..........
Synoviae    .......TTA TCT--...GA ..-------- ------.... .C.A....TT ..........
Galnaceu    .....AACGT TCT--.G.GA ..-------- ------.... .C........ ..........

                                                                                                 1200
Ms01        TGGGGATGAC GTCAAATCAT CATGCCTCTT ACGAGTGGGG CCACACACGT GCTACAATGG
Ms02        ......C... .......... .......... .......... .A........ ..........
Ms03        ......C... .......... .......... .......... .A........ ..........
Synoviae    ......C... .......... .......... .......... .A........ ..........
Galnaceu    ......C... .......... .......... .......... .A........ ..........

                                                                                                 1260
Ms01        TCGGTACAAA GAGAAGCAAT ATGGCGACAT GGAGCAAATC TCAAAAAGCC GATCTCAGTT
Ms02        A......... .......... .C......G. T......... .......A.. .T........
Ms03        AT........ .......... .C......G. .......... .......A.. AT........
Synoviae    A..A...... .........A ..A.T..T.. CA........ .......ATT CG........
Galnaceu    AT........ ....C..... .C......G. .......... .......A.. AT........

                                                                                                 1320
Ms01        CGGATTGGAG TCTGCAATTC GACTCCATGA AGTCGGAATC GCTAGTAATC GCAGATCAGC
Ms02        .......T.. .......C.. ....A..... .......... .......... .T........
Ms03        .......T.. .......C.. ....A..... .......... .......... .T........
Synoviae    .......T.. .......C.. ....A..... .......... .......... .T........
Galnaceu    .......T.. .......C.. ....A..... .......... .......... .T......CG
```

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                                                                      1380
Ms01        TATGCTGCGG TGAATACGTT CTCGGGTCTT GTACACACCG CCCGTCACAC CATGGGAGCT
Ms02        ..C...A... .......... .......... .......... .......... ..........
Ms03        ..C...A... .......... .......... .......... .......... ..........
Synoviae    ..C...A... .......... .......... .......... .......... ..........
Galnaceu    ..C...A... .......... .......... .......... .......... ..........

                                                                      1440
Ms01        GGTAATACCC AAAGTCGGTT TGCTAACCTC GGAGGCGACC GCTCTAAGGT AGGACTGGTG
Ms02        ......G... G......... .TG.T.A..A C.GA.AC.A. TGC......C .....C....
Ms03        ......G... G......... .TG.T.A..A C.GAAAC.A. TGC......C ..........
Synoviae    ......G... G......... ..T....T.. ....A....- TGC......C .....C....
Galnaceu    ......G... G......... .TG.T.A..A C.GA.AC.A. TGC......C ..........

Ms01        ACTGGGGT
Ms02        ........-
Ms03        ........-
Synoviae    ........-
Galnaceu    ........-
```

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1. STATISTICAL ANALYSIS OF VACCINATION TRIAL USING LIVE La Sota VACCINE

1.1. Statistical analysis:

The SAS System

General Linear Models Procedure

Class Level Information

Class	Levels	Values
TRT	2	1 2
TIME	6	0 7 14 21 28 35

Number of observations in data set = 138

NOTE: Due to missing values, only 126 observations can be used in this analysis.

Dependent Variable: RESP

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	11	0.22508535	0.02046230	9.96	<.0001
Error	113	0.23226150	0.00205541		
Corrected Total	124	0.45734685			

R-Square		C.V.	Root MSE	RESP Mean	
0.492155		75.68217	0.045337	0.059904	

Source	DF	Type I SS	Mean Square	F Value	Pr > F
TRT	1	0.00166717	0.00166717	0.81	0.3697
TIME	5	0.18576952	0.03715390	18.08	<.0001
TRT*TIME	5	0.03764866	0.00752973	3.66	0.0041

1.1.1. Input data:

The input data on each page is arranged in two columns (each containing treatment (trt), time and response (resp)), from top to bottom and left to right.

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TRT	TIME	RESP
1	0	0.000
1	0	0.000
1	0	0.030
1	0	0.007
1	0	0.024
1	0	0.000
1	0	0.018
1	0	0.005
1	0	0.000
1	7	0.194
1	7	0.002
1	7	0.014
1	7	0.021
1	7	0.013
1	7	0.016
1	7	0.043
1	7	0.041
1	7	0.070
1	14	0.033
1	14	0.056
1	14	0.032
1	14	0.032
1	14	0.079
1	14	0.060
1	14	0.059
1	14	0.014
1	14	0.030
1	21	0.035
1	21	0.044
1	21	0.075
1	21	0.078
1	21	0.111
1	21	0.005
1	21	0.034
1	21	0.063
1	21	0.074
1	28	0.065
1	28	0.120
1	28	0.057
1	28	0.089
1	28	0.085
1	28	0.090
1	28	0.010
1	28	.
1	28	0.030
1	35	0.136
1	35	0.253
1	35	0.196
1	35	0.080
1	35	0.086
1	35	0.010
1	35	0.036
1	35	0.135
2	0	0.070
2	0	0.075
2	0	0.034
2	0	0.054
2	0	0.070
2	0	0.140
2	0	0.004
2	0	0.047
2	0	0.052
2	0	0.034
2	0	0.070
2	0	0.046
2	0	0.127
2	0	0.076
2	7	0.007
2	7	0.000
2	7	0.023
2	7	0.002
2	7	0.003
2	7	0.000
2	7	0.009
2	7	0.011
2	7	0.003
2	7	0.112
2	7	0.003
2	7	0.016
2	7	0.012
2	7	0.000
2	14	0.043
2	14	0.004
2	14	0.053
2	14	0.009
2	14	0.022
2	14	0.015
2	14	0.010
2	14	0.081
2	14	0.000
2	14	0.007
2	14	0.007
2	14	0.098
2	14	0.010
2	14	0.196
2	21	0.072
2	21	0.000
2	21	0.091
2	21	0.000
2	21	0.000
2	21	0.000
2	21	0.108
2	21	.
2	21	0.139
2	21	0.006
2	21	0.017
2	21	0.004
2	21	0.041
2	21	0.007
2	28	0.081
2	28	.
2	28	0.095
2	28	.
2	28	.
2	28	0.109
2	28	.
2	28	.
2	28	0.134
2	28	0.160
2	28	0.020
2	28	0.038
2	28	0.083
2	28	0.178
2	35	0.215
2	35	.
2	35	.
2	35	0.188
2	35	.
2	35	0.141
2	35	.
2	35	.
2	35	0.167
2	35	0.191
2	35	0.095
2	35	0.170
2	35	0.270
2	35	0.123

Treatment:

1 = Control group

2 = Vaccinated group

Time = Days after vaccination

Response = ELISA titres

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2. STATISTICAL ANALYSIS OF ANTI-*PLASMODIUM* IMMUNE RESPONSE OF PENGUINS THAT WERE RELEASED VS PENGUINS THAT DIED DURING REHABILITATION

2.1. Released vs Died: October 2001 to November 2001

The SAS System

General Linear Models Procedure

Class Level Information

Class	Levels	Values
TRT	2	1 2
TIME	3	1 2 3

Number of observations in data set = 156

NOTE: Due to missing values, only 144 observations can be used in this analysis.

Dependent Variable: RESP

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	5	2.26814407	0.45362881	1.59	0.1655
Error	138	39.2524549	0.28443808		
Corrected Total	143	41.52059866			

R-Square		C.V.	Root MSE	RESP Mean	
0.054627		136.1132	0.533327	0.391826	

Source	DF	Type I SS	Mean Square	F Value	Pr > F
TRT	1	0.27469311	0.27469311	0.97	0.3275
TIME	2	1.65196159	0.82598079	2.90	0.0582
TRT*TIME	2	0.34148937	0.17074469	0.60	0.5501

2.1.1. Input data:

The input data on each page is arranged in two columns (each containing treatment (trt), time and response (resp)), from top to bottom and left to right.

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TRT	TIME	RESP	TRT	TIME	RESP	TRT	TIME	RESP
1	1	0.215.	1	1	0.150	1	1	0.387
1	2	0.098	1	2	0.084	1	2	0.401
1	3	0.151.	1	3	0.182	1	3	0.152
1	1	0.296	1	1	0.046	1	1	0.169
1	2	0.170	1	2	0.120	1	2	0.357
1	3	0.228	1	3	.	1	3	1.130
1	1	2.827	1	1	0.169	1	1	0.125
1	2	2.373	1	2	0.358	1	2	0.297
1	3	.	1	3	.	1	3	.
1	1	0.182	1	1	0.122	1	1	0.132
1	2	0.154	1	2	0.298	1	2	0.143
1	3	0.219	1	3	1.280	1	3	.
1	1	0.206	1	1	0.086	1	1	0.220
1	2	0.104	1	2	0.316	1	2	0.295
1	3	0.237	1	3	2.850	1	3	0.448
1	1	0.189	1	1	0.167	1	1	0.258
1	2	0.024	1	2	0.128	1	2	0.082
1	3	0.101	1	3	0.077	1	3	0.500
1	1	0.296	1	1	0.188	1	1	0.128
1	2	0.161	1	2	0.463	1	2	0.120
1	3	0.420	1	3	0.238	1	3	0.169
1	1	0.287	1	1	0.250	1	1	0.227
1	2	0.288	1	2	0.252	1	2	0.194
1	3	0.440	1	3	0.466	1	3	1.440
1	1	0.220	1	1	0.134	1	1	0.454
1	2	0.047	1	2	0.454	1	2	0.058
1	3	.	1	3	0.231	1	3	.
1	1	0.216	1	1	0.198	1	1	0.214
1	2	2.362	1	2	0.151	1	2	0.714
1	3	2.764	1	3	0.362	1	3	.
1	1	0.199	1	1	0.515	2	1	0.119
1	2	0.125	1	2	2.899	2	2	0.463
1	3	0.137	1	3	1.439	2	3	0.318
1	1	0.296	1	1	0.193	2	1	0.105
1	2	0.362	1	2	0.155	2	2	0.320
1	3	.	1	3	0.272	2	3	0.126
1	1	0.060	1	1	0.168	2	1	0.347
1	2	0.308	1	2	0.212	2	2	0.026
1	3	0.237	1	3	0.220	2	3	0.135

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TRT	TIME	RESP
2	1	0.385
2	2	0.108
2	3	0.317
2	1	0.600
2	2	0.181
2	3	0.313
2	1	0.385
2	2	0.319
2	3	0.999
2	1	0.130
2	2	0.213
2	3	0.497
2	1	0.393
2	2	0.310
2	3	0.715
2	1	0.601
2	2	0.272
2	3	0.706
2	1	0.301
2	2	0.300
2	3	.
2	1	0.186
2	2	0.366
2	3	0.219
2	1	0.325
2	2	0.239
2	3	0.257
2	1	0.688
2	2	0.129
2	3	.
2	1	0.346
2	2	0.144
2	3	0.229
2	1	0.079
2	2	0.186
2	3	1.079
2	1	0.194
2	2	0.047
2	3	.

Treatment:

1 = Released group

2 = Died group

Time = Weeks after admission

Response = ELISA titres

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2.2. Released vs Died: December 2001 to January 2002

The SAS System

General Linear Models Procedure

Class Level Information

Class	Levels	Values
TRT	2	1 2
TIME	3	1 2 3

Number of observations in data set = 144

NOTE: Due to missing values, only 130 observations can be used in this analysis.

Dependent Variable: RESP

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	5	2.69429117	0.53885823	2.89	0.0168
Error	124	23.13150194.	0.18654437		
Corrected Total	129	25.82579311			

R-Square		C.V.	Root MSE	RESP Mean	
0.104326		147.2387	0.431908	0.293338	

Source	DF	Type I SS	Mean Square	F Value	Pr > F
TRT	1	0.09602062	0.09602062	0.51	0.4744
TIME	2	2.02432943	1.01216472	5.43	0.0055
TRT*TIME	2	0.57394111	0.28697056	1.54	0.2188

2.2.1. Input data:

The input data on each page is arranged in two columns (each containing trt, time and resp), from top to bottom and left to right.

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TRT	TIME	RESP	TRT	TIME	RESP	TRT	TIME	RESP
1	1	0.107	1	2	1.365	2	3	0.084
1	2	0.434	1	3	1.156			
1	3	.				2	1	0.087
			1	1	0.028	2	2	0.092
1	1	0.046	1	2	0.091	2	3	.
1	2	1.850	1	3	.			
1	3	0.526				2	1	0.170
			1	1	0.222	2	2	0.236
1	1	0.042	1	2	0.292	2	3	0.062
1	2	0.109	1	3	0.613			
1	3	1.737				2	1	0.012
			1	1	0.120	2	2	0.091
1	1	0.039	1	2	0.121	2	3	2.017
1	2	0.062	1	3	0.159			
1	3	.				2	1	0.005
			1	1	0.164	2	2	0.034
1	1	0.179	1	2	0.224	2	3	.
1	2	0.327	1	3	0.141			
1	3	0.193				2	1	0.207
			1	1	0.068	2	2	0.133
1	1	0.093	1	2	0.055	2	3	.
1	2	0.265	1	3	0.465			
1	3	1.748				2	1	0.109
			1	1	0.066	2	2	0.126
1	1	0.123	1	2	0.195	2	3	0.146
1	2	0.098	1	3	0.369			
1	3	0.025				2	1	0.061
			1	1	0.079	2	2	0.175
1	1	0.033	1	2	0.101	2	3	0.245
1	2	0.047	1	3	0.101			
1	3	.				2	1	0.046
			1	1	0.083	2	2	0.313
1	1	0.123	1	2	0.095	2	3	2.511
1	2	0.468	1	3	0.134			
1	3	0.496				2	1	0.147
			1	1	0.143	2	2	0.200
1	1	0.269	1	2	1.806	2	3	0.405
1	2	1.033	1	3	.			
1	3	0.397				2	1	0.162
			1	1	0.193	2	2	0.218
1	1	0.049	1	2	0.248	2	3	0.092
1	2	0.146	1	3	0.196			
1	3	0.042				2	1	0.229
			1	1	0.067	2	2	0.141
1	1	0.033	1	2	0.058	2	3	0.332
1	2	0.048	1	3	0.076			
1	3	0.067				2	1	0.000
			2	1	0.117	2	2	0.174
1	1	0.071	2	2	0.027	2	3	.
1	2	0.073	2	3	.			
1	3	0.051				2	1	0.157
			2	1	0.037	2	2	0.353
1	1	1.552	2	2	0.079	2	3	.

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2.3. Released vs Died: February 2002 to March 2002

The SAS System

General Linear Models Procedure

Class Level Information

Class	Levels	Values
TRT	2	1 2
TIME	3	1 2 3

Number of observations in data set = 30

NOTE: Due to missing values, only 26 observations can be used in this analysis.

Dependent Variable: RESP

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	5	0.46046108	0.09209222	2.42	0.0720
Error	20	0.76183942.	0.03809197		
Corrected Total	25	1.22230050			

R-Square		C.V.	Root MSE	RESP Mean	
0.376717		75.50160	0.195172	0.258500	

Source	DF	Type I SS	Mean Square	F Value	Pr > F
TRT	1	0.07732246	0.07732246	2.03	0.1697
TIME	2	0.28404605	0.14202303	3.73	0.0421
TRT*TIME	2	0.09909257	0.04954628	1.30	0.2944

2.3.1. Input data:

The input data on each page is arranged in two columns (each containing trt, time and resp), from top to bottom and left to right.

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2.4. Released vs Died: April 2002 to May 2002

The SAS System

General Linear Models Procedure

Class Level Information

Class	Levels	Values
TRT	2	1 2
TIME	3	1 2 3

Number of observations in data set = 198

NOTE: Due to missing values, only 185 observations can be used in this analysis.

Dependent Variable: RESP

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	5	0.38328266	0.07665653	2.36	0.0418
Error	179	5.81089088.	0.03246308		
Corrected Total	184	6.19417354			

R-Square		C.V.	Root MSE	RESP Mean	
0.061878		194.2334	0.180175	0.092762	

Source	DF	Type I SS	Mean Square	F Value	Pr > F
TRT	1	0.10891046	0.10891046	3.35	0.0687
TIME	2	0.21463628	0.10731814	3.31	0.0389
TRT*TIME	2	0.05973592	0.02986796	0.92	0.4004

2.4.1. Input data:

The input data on each page is arranged in two columns (each containing trt, time and resp), from top to bottom and left to right.

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TRT	TIME	RESP	TRT	TIME	RESP	TRT	TIME	RESP
1	1	1.125	1	2	0.115	1	3	0.091
1	2	0.246	1	3	0.085			
1	3	0.213				1	1	0.051
			1	1	0.027	1	2	0.104
1	1	0.141	1	2	0.042	1	3	0.061
1	2	0.034	1	3	0.071			
1	3	0.039				1	1	0.080
			1	1	0.028	1	2	0.035
1	1	0.247	1	2	0.075	1	3	.
1	2	0.027	1	3	0.050			
1	3	0.036				1	1	0.049
			1	1	0.016	1	2	0.052
1	1	0.051	1	2	0.044	1	3	.
1	2	0.059	1	3	0.021			
1	3	0.249				1	1	0.091
			1	1	0.017	1	2	0.338
1	1	0.035	1	2	0.017	1	3	0.097
1	2	0.154	1	3	0.019			
1	3	.				1	1	0.065
			1	1	0.031	1	2	0.126
1	1	0.028	1	2	0.059	1	3	0.060
1	2	0.052	1	3	0.025			
1	3	0.045				1	1	0.103
			1	1	0.044	1	2	0.064
1	1	0.020	1	2	0.020	1	3	0.021
1	2	0.037	1	3	.			
1	3	0.057				1	1	0.040
			1	1	0.029	1	2	0.141
1	1	0.049	1	2	0.034	1	3	0.049
1	2	0.088	1	3	0.054			
1	3	0.064				1	1	0.090
			1	1	0.032	1	2	0.021
1	1	0.047	1	2	0.107	1	3	0.061
1	2	0.106	1	3	0.101			
1	3	.				1	1	0.226
			1	1	0.020	1	2	0.052
1	1	0.079	1	2	0.152	1	3	0.070
1	2	0.084	1	3	2.032			
1	3	0.113				1	1	0.057
			1	1	0.030	1	2	0.032
1	1	0.027	1	2	0.077	1	3	0.068
1	2	0.077	1	3	0.067			
1	3	.				1	1	0.026
			1	1	0.063	1	2	0.059
1	1	0.028	1	2	0.040	1	3	0.041
1	2	0.058	1	3	0.029			
1	3	.				1	1	0.030
			1	1	0.028	1	2	0.075
1	1	0.011	1	2	0.038	1	3	0.034
1	2	0.061	1	3	0.278			
1	3	0.023				1	1	0.046
			1	1	0.068	1	2	0.011
1	1	0.020	1	2	0.080	1	3	0.053

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TRT	TIME	RESP	TIME	TRT	RESP
1	1	0.020	1	1	0.028
1	2	0.003	1	2	0.027
1	3	0.070	1	3	0.093
1	1	0.026	1	1	0.027
1	2	0.027	1	2	0.053
1	3	0.070	1	3	0.222
1	1	0.044	1	1	0.021
1	2	0.038	1	2	0.069
1	3	0.043	1	3	0.045
1	1	0.068	1	1	0.058
1	2	0.118	1	2	0.045
1	3	.	1	3	0.060
1	1	0.023	1	1	0.022
1	2	0.063	1	2	0.021
1	3	0.122	1	3	0.342
1	1	0.019	1	1	0.047
1	2	0.032	1	2	0.033
1	3	0.061	1	3	0.043
1	1	0.014	2	1	0.016
1	2	0.024	2	2	0.037
1	3	0.070	2	3	.
1	1	0.020	2	1	0.015
1	2	0.063	2	2	0.067
1	3	0.074	2	3	0.085
1	1	0.016	2	1	0.083
1	2	0.011	2	2	0.105
1	3	0.366	2	3	.
1	1	0.004	2	1	0.071
1	2	0.013	2	2	0.122
1	3	0.086	2	3	0.618
1	1	0.010	2	1	0.127
1	2	0.090	2	2	0.514
1	3	0.092	2	3	.
1	1	0.016	2	1	0.311
1	2	0.013	2	2	0.109
1	3	0.048	2	3	0.219
1	1	0.045	2	1	0.052
1	2	0.028	2	2	0.089
1	3	0.043	2	3	0.360

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2.5. Released vs Died: June 2002 to July 2002

The SAS System

General Linear Models Procedure

Class Level Information

Class	Levels	Values
TRT	2	1 2
TIME	3	1 2 3

Number of observations in data set = 153

NOTE: Due to missing values, only 151 observations can be used in this analysis.

Dependent Variable: RESP

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	5	0.39007432	0.07801486	2.85	0.0173
Error	145	3.96442906	0.02734089		
Corrected Total	150	4.35450338			

R-Square		C.V.	Root MSE	RESP Mean	
0.089580		169.6770	0.165351	0.097450	

Source	DF	Type I SS	Mean Square	F Value	Pr > F
TRT	1	0.00322211	0.00322211	0.12	0.7319
TIME	2	0.37257128	0.18628564	6.81	0.0015
TRT*TIME	2	0.01428093	0.00714047	0.26	0.7705

2.5.1. Input data:

The input data on each page is arranged in two columns (each containing trt, time and resp), from top to bottom and left to right.

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TRT	TIME	RESP	TRT	TIME	RESP	TRT	TIME	RESP
1	1	0.021	1	2	0.239	1	3	0.370
1	2	0.078	1	3	0.051			
1	3	0.042				1	1	0.073
			1	1	0.063	1	2	0.000
1	1	0.037	1	2	0.061	1	3	0.548
1	2	0.065	1	3	0.145			
1	3	0.069				1	1	0.067
			1	1	0.077	1	2	0.000
1	1	0.041	1	2	0.081	1	3	0.976
1	2	0.135	1	3	0.064			
1	3	0.035				1	1	0.112
			1	1	0.112	1	2	0.048
1	1	0.085	1	2	0.172	1	3	0.010
1	2	0.050	1	3	1.394			
1	3	0.053				1	1	0.021
			1	1	0.066	1	2	0.034
1	1	0.081	1	2	0.033	1	3	0.082
1	2	0.049	1	3	0.005			
1	3	0.276				1	1	0.000
			1	1	0.121	1	2	0.019
1	1	0.040	1	2	0.117	1	3	.
1	2	0.022	1	3	0.025			
1	3	0.081				1	1	0.030
			1	1	0.144	1	2	0.023
1	1	0.059	1	2	0.120	1	3	0.046
1	2	0.048	1	3	0.340			
1	3	0.108				1	1	0.034
			1	1	0.065	1	2	0.037
1	1	0.034	1	2	0.046	1	3	0.044
1	2	0.094	1	3	0.011			
1	3	0.088				1	1	0.021
			1	1	0.092	1	2	0.057
1	1	0.063	1	2	0.052	1	3	0.101
1	2	0.107	1	3	0.341			
1	3	0.063				1	1	0.035
			1	1	0.153	1	2	0.029
1	1	0.040	1	2	0.092	1	3	0.042
1	2	0.042	1	3	0.534			
1	3	0.080				1	1	0.027
			1	1	0.130	1	2	0.068
1	1	0.032	1	2	0.049	1	3	0.054
1	2	0.106	1	3	0.975			
1	3	0.016				1	1	0.070
			1	1	0.124	1	2	0.061
1	1	0.019	1	2	0.052	1	3	0.040
1	2	0.069	1	3	0.000			
1	3	0.118				1	1	0.067
			1	1	0.100	1	2	0.082
1	1	0.073	1	2	0.002	1	3	0.042
1	2	0.046	1	3	0.316			
1	3	0.053				1	1	0.062
			1	1	0.182	1	2	0.025
1	1	0.044	1	2	0.000	1	3	0.023

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2.6. Released vs Died: August 2002 to September 2002

The SAS System

General Linear Models Procedure

Class Level Information

Class	Levels	Values
TRT	2	1 2
TIME	3	1 2 3

Number of observations in data set = 183

NOTE: Due to missing values, only 171 observations can be used in this analysis.

Dependent Variable: RESP

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	4	0.13639100	0.03409775	1.60	0.1769
Error	166	3.53934196	0.02132134		
Corrected Total	170	3.67573296			

R-Square		C.V.	Root MSE	RESP Mean	
0.037106		287.9281	0.146018	0.050713	

Source	DF	Type I SS	Mean Square	F Value	Pr > F
TRT	1	0.00392680	0.00392680	0.18	0.6684
TIME	2	0.13244705	0.06622352	3.11	0.0474
TRT*TIME	1	0.00001716	0.00001716	0.00	0.9774

2.6.1. Input data:

The input data on each page is arranged in two columns (each containing trt, time and resp), from top to bottom and left to right.

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TRT	TIME	RESP
1	1	0.000
1	2	0.072
1	3	.
1	1	0.000
1	2	0.035
1	3	0.085
1	1	0.006
1	2	0.037
1	3	0.049
1	1	0.000
1	2	0.114
1	3	0.090
1	1	0.005
1	2	0.021
1	3	0.021
1	1	0.000
1	2	0.052
1	3	0.084
1	1	0.074
1	2	0.075
1	3	0.118
1	1	0.102
1	2	0.048
1	3	0.034
1	1	0.075
1	2	0.046
1	3	1.675
1	1	0.040
1	2	0.011
1	3	0.077
1	1	0.006
1	2	0.046
1	3	0.002
1	1	0.032
1	2	0.052
1	3	0.027
1	1	0.024
1	2	0.010
1	3	0.040
1	1	0.018

TRT	TIME	RESP
1	2	0.029
1	3	0.125
1	1	0.056
1	2	0.002
1	3	0.024
1	1	0.014
1	2	0.014
1	3	0.026
1	1	0.034
1	2	0.007
1	3	0.929
1	1	0.026
1	2	0.030
1	3	0.045
1	1	0.040
1	2	0.046
1	3	0.301
1	1	0.044
1	2	0.079
1	3	0.108
1	1	0.029
1	2	0.044
1	3	0.043
1	1	0.026
1	2	0.173
1	3	0.023
1	1	0.040
1	2	0.029
1	3	0.010
1	1	0.069
1	2	0.047
1	3	.
1	1	0.039
1	2	0.030
1	3	0.007
1	1	0.037
1	2	0.124
1	3	0.018
1	1	0.011
1	2	0.021

TRT	TIME	RESP
1	3	0.020
1	1	0.055
1	2	0.009
1	3	0.016
1	1	0.086
1	2	0.002
1	3	0.014
1	1	0.029
1	2	0.002
1	3	0.005
1	1	0.053
1	2	0.002
1	3	0.027
1	1	0.067
1	2	0.025
1	3	0.006
1	1	0.028
1	2	0.037
1	3	0.002
1	1	0.025
1	2	0.002
1	3	.
1	1	0.017
1	2	0.007
1	3	0.006
1	1	0.002
1	2	0.027
1	3	.
1	1	0.007
1	2	0.000
1	3	0.032
1	1	0.000
1	2	0.008
1	3	0.034
1	1	0.000
1	2	0.004
1	3	0.016
1	1	0.021
1	2	0.030
1	3	.

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TRT	TIME	RESP
1	1	0.004
1	2	0.030
1	3	.
1	1	0.001
1	2	0.063
1	3	0.013
1	1	0.009
1	2	0.029
1	3	0.016
1	1	0.038
1	2	0.017
1	3	0.008
1	1	0.002
1	2	0.031
1	3	.
1	1	0.000
1	2	0.035
1	3	.
1	1	0.037
1	2	0.159
1	3	0.043
1	1	0.040
1	2	0.053
1	3	0.022
1	1	0.036
1	2	0.035
1	3	0.007
1	1	0.015
1	2	0.028
1	3	0.037
1	1	0.020
1	2	0.024
1	3	0.105
1	1	0.055
1	2	0.084
1	3	.
1	1	0.036
1	2	0.022
1	3	.
1	1	0.036

TRT	TIME	RESP
1	2	0.027
1	3	0.047
1	1	0.015
1	2	0.014
1	3	0.051
1	1	0.042
1	2	0.037
1	3	0.058
1	1	0.023
1	2	0.076
1	3	0.041
1	1	0.023
1	2	0.038
1	3	0.022
1	1	0.021
1	2	0.012
1	3	0.031
2	1	0.010
2	2	0.023
2	3	.
2	1	0.024
2	2	0.022
2	3	.

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2.7. Released vs Died: October 2002 to November 2002

The SAS System

General Linear Models Procedure

Class Level Information

Class	Levels	Values
TRT	2	1 2
TIME	3	1 2 3

Number of observations in data set = 126

NOTE: Due to missing values, only 116 observations can be used in this analysis.

Dependent Variable: RESP

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	5	0.00147919	0.00029584	0.31	0.9052
Error	110	0.10452373	0.00095022		
Corrected Total	115	0.10600292			

R-Square		C.V.	Root MSE	RESP Mean	
0.013954		93.48408	0.030826	0.032974	

Source	DF	Type I SS	Mean Square	F Value	Pr > F
TRT	1	0.00041462	0.00041462	0.44	0.5103
TIME	2	0.00028035	0.00014018	0.15	0.8630
TRT*TIME	2	0.00078421	0.00039211	0.41	0.6629

2.7.1. Input data:

The input data on each page is arranged in two columns (each containing trt, time and resp), from top to bottom and left to right.

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TRT	TIME	RESP	TRT	TIME	RESP	TRT	TIME	RESP
1	1	0.043	1	2	0.008	1	3	.
1	2	0.031	1	3	0.012			
1	3	.				1	1	0.070
			1	1	0.000	1	2	0.027
1	1	0.020	1	2	0.013	1	3	0.040
1	2	0.039	1	3	0.050			
1	3	0.008				1	1	0.055
			1	1	0.006	1	2	0.044
1	1	0.021	1	2	0.043	1	3	.
1	2	0.036	1	3	0.027			
1	3	.				1	1	0.013
			1	1	0.002	1	2	0.017
1	1	0.021	1	2	0.032	1	3	0.020
1	2	0.003	1	3	0.018			
1	3	0.014				1	1	0.026
			1	1	0.050	1	2	0.056
1	1	0.039	1	2	0.013	1	3	.
1	2	0.010	1	3	0.005			
1	3	0.009				1	1	0.049
			1	1	0.118	1	2	0.018
1	1	0.067	1	2	0.108	1	3	0.024
1	2	0.075	1	3	0.035			
1	3	0.002				1	1	0.024
			1	1	0.017	1	2	0.020
1	1	0.015	1	2	0.018	1	3	0.013
1	2	0.031	1	3	0.010			
1	3	0.002				1	1	0.014
			1	1	0.040	1	2	0.028
1	1	0.114	1	2	0.024	1	3	0.051
1	2	0.072	1	3	0.019			
1	3	0.073				1	1	0.021
			1	1	0.030	1	2	0.056
1	1	0.069	1	2	0.020	1	3	0.235
1	2	0.008	1	3	0.040			
1	3	0.055				2	1	0.018
			1	1	0.032	2	2	0.081
1	1	0.025	1	2	0.071	2	3	.
1	2	0.002	1	3	0.043			
1	3	0.030				2	1	0.070
			1	1	0.027	2	2	0.045
1	1	0.002	1	2	0.022	2	3	.
1	2	0.011	1	3	.			
1	3	0.007				2	1	0.000
			1	1	0.027	2	2	0.016
1	1	0.014	1	2	0.055	2	3	0.033
1	2	0.057	1	3	0.025			
1	3	0.066				2	1	0.000
			1	1	0.044	2	2	0.033
1	1	0.000	1	2	0.036	2	3	0.020
1	2	0.011	1	3	0.028			
1	3	0.031				2	1	0.020
			1	1	0.029	2	2	0.033
1	1	0.000	1	2	0.047	2	3	0.038

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2.8. Released vs Died: December 2002 to January 2003

The SAS System

General Linear Models Procedure

Class Level Information

Class	Levels	Values
TRT	2	1 2
TIME	3	1 2 3

Number of observations in data set = 96

NOTE: Due to missing values, only 89 observations can be used in this analysis.

Dependent Variable: RESP

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	5	0.13904117	0.02780823	1.53	0.1901
Error	83	1.51098932	0.01820469		
Corrected Total	88	1.65003049			

R-Square		C.V.	Root MSE	RESP Mean	
0.084266		185.7433	0.134925	0.072640	

Source	DF	Type I SS	Mean Square	F Value	Pr > F
TRT	1	0.011867745	0.01186745	0.65	0.4217
TIME	2	0.10036332	0.05018166	2.76	0.0693
TRT*TIME	2	0.02681040	0.01340520	0.74	0.4820

2.8.1. Input data:

The input data on each page is arranged in two columns (each containing trt, time and resp), from top to bottom and left to right.

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TRT	TIME	RESP
1	1	0.004
1	2	0.050
1	3	0.005
1	1	0.000
1	2	0.026
1	3	0.000
1	1	0.037
1	2	0.025
1	3	0.000
1	1	0.059
1	2	0.039
1	3	0.000
1	1	0.020
1	2	0.010
1	3	0.000
1	1	0.003
1	2	0.000
1	3	0.009
1	1	0.002
1	2	0.000
1	3	0.017
1	1	0.009
1	2	0.000
1	3	0.035
1	1	0.002
1	2	0.006
1	3	0.039
1	1	0.004
1	2	0.023
1	3	0.062
1	1	0.005
1	2	0.013
1	3	0.091
1	1	0.018
1	2	0.102
1	3	.
1	1	0.029
1	2	0.074
1	3	.
1	1	0.013
1	2	0.089
1	3	.
1	1	0.083
1	2	0.055
1	3	0.005
1	1	0.045
1	2	0.021
1	3	0.010
1	1	0.024
1	2	0.085
1	3	0.015
1	1	0.034
1	2	0.058
1	3	0.067
1	1	0.046
1	2	0.007
1	3	0.027
1	1	0.085
1	2	0.021
1	3	0.021
1	1	0.134
1	2	0.253
1	3	0.683
1	1	0.092
1	2	0.709
1	3	0.609
2	1	0.004
2	2	0.000
2	3	.
2	1	0.000
2	2	0.358
2	3	0.064
2	1	0.000
2	2	0.010
2	3	.
2	1	0.008
2	2	0.055
2	3	0.373
2	1	0.001
2	2	0.013
2	3	0.073
2	1	0.031
2	2	0.222
2	3	.
2	1	0.035
2	2	0.067
2	3	0.013
2	1	0.021
2	2	0.088
2	3	.
2	1	0.022
2	2	0.126
2	3	0.097
2	1	0.117
2	2	0.110
2	3	0.448

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3. STATISTICAL ANALYSIS OF ANTI-*PLASMODIUM* IMMUNE RESPONSE OF PENGUINS THAT WERE OILED VS PENGUINS THAT WERE NOT OILED

3.1. Oiled vs non-Oiled: October 2001 to November 2001

The SAS System

General Linear Models Procedure

Class Level Information

Class	Levels	Values
TRT	2	1 2
TIME	3	1 2 3

Number of observations in data set = 156

NOTE: Due to missing values, only 144 observations can be used in this analysis.

Dependent Variable: RESP

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	5	2.37383604	0.47476721	1.67	0.1449
Error	138	39.14676262	0.28367219		
Corrected Total	143	41.52059866			

R-Square		C.V.	Root MSE	RESP Mean	
0.057172		135.9298	0.532609	0.391826	

Source	DF	Type I SS	Mean Square	F Value	Pr > F
TRT	1	0.15179615	0.15179615	0.54	0.4657
TIME	2	1.58162244	0.79081122	2.79	0.0650
TRT*TIME	2	0.64041745	0.32020873	1.13	0.3264

3.1.1. Input data:

The input data on each page is arranged in two columns (each containing treatment (trt), time and response (resp)), from top to bottom and left to right.

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TRT	TIME	RESP	TRT	TIME	RESP	TRT	TIME	RESP
1	1	0.215	1	2	0.084	1	3	0.152
1	2	0.098	1	3	0.182			
1	3	0.151				2	1	0.169
			2	1	0.046	2	2	0.357
1	1	0.296	2	2	0.120	2	3	1.130
1	2	0.170	2	3	.			
1	3	0.228				2	1	0.125
			1	1	0.169	2	2	0.297
2	1	2.827	1	2	0.358	2	3	.
2	2	2.373	1	3	.			
2	3	.				1	1	0.132
			1	1	0.122	1	2	0.143
2	1	0.182	1	2	0.298	1	3	.
2	2	0.154	1	3	1.280			
2	3	0.219				2	1	0.220
			1	1	0.086	2	2	0.295
2	1	0.206	1	2	0.316	2	3	0.448
2	2	0.104	1	3	2.850			
2	3	0.237				2	1	0.258
			1	1	0.167	2	2	0.082
2	1	0.189	1	2	0.128	2	3	0.500
2	2	0.024	1	3	0.077			
2	3	0.101				2	1	0.128
			1	1	0.188	2	2	0.120
2	1	0.296	1	2	0.463	2	3	0.169
2	2	0.161	1	3	0.238			
2	3	0.420				1	1	0.227
			1	1	0.250	1	2	0.194
2	1	0.287	1	2	0.252	1	3	1.440
2	2	0.288	1	3	0.466			
2	3	0.440				2	1	0.454
			2	1	0.134	2	2	0.058
1	1	0.220	2	2	0.454	2	3	.
1	2	0.047	2	3	0.231			
1	3	.				2	1	0.214
			2	1	0.198	2	2	0.714
1	1	0.216	2	2	0.151	2	3	.
1	2	2.362	2	3	0.362			
1	3	2.764				2	1	0.119
			1	1	0.515	2	2	0.463
1	1	0.199	1	2	2.899	2	3	0.318
1	2	0.125	1	3	1.439			
1	3	0.137				1	1	0.105
			1	1	0.193	1	2	0.320
2	1	0.296	1	2	0.155	1	3	0.126
2	2	0.362	1	3	0.272			
2	3	.				1	1	0.347
			1	1	0.168	1	2	0.026
1	1	0.060	1	2	0.212	1	3	0.135
1	2	0.308	1	3	0.220			
1	3	0.237				1	1	0.385
			1	1	0.387	1	2	0.108
1	1	0.150	1	2	0.401	1	3	0.317

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TRT	TIME	RESP
1	1	0.600
1	2	0.181
1	3	0.313
1	1	0.385
1	2	0.319
1	3	0.999
1	1	0.130
1	2	0.213
1	3	0.497
1	1	0.393
1	2	0.310
1	3	0.715
1	1	0.601
1	2	0.272
1	3	0.706
1	1	0.301
1	2	0.300
1	3	.
2	1	0.186
2	2	0.366
2	3	0.219
1	1	0.325
1	2	0.239
1	3	0.257
1	1	0.688
1	2	0.129
1	3	.
1	1	0.346
1	2	0.144
1	3	0.229
1	1	0.079
1	2	0.186
1	3	1.079
2	1	0.194
2	2	0.047
2	3	.

Treatment:

1 = Non-oiled group

2 = Oiled group

Time = Weeks after admission

Response = ELISA titres

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3.2. Oiled vs non-Oiled: December 2001 to January 2002

The SAS System

General Linear Models Procedure

Class Level Information

Class	Levels	Values
TRT	2	1 2
TIME	3	1 2 3

Number of observations in data set = 144

NOTE: Due to missing values, only 130 observations can be used in this analysis.

Dependent Variable: RESP

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	5	3.67158250	0.73431650	4.11	0.0017
Error	124	22.15421061	0.17866299		
Corrected Total	129	25.82579311			

R-Square		C.V.	Root MSE	RESP Mean	
0.142167		144.0948	0.422685	0.293338	

Source	DF	Type I SS	Mean Square	F Value	Pr > F
TRT	1	0.01519940	0.01519940	0.09	0.7710
TIME	2	2.06571358	1.03285679	5.78	0.0040
TRT*TIME	2	1.59066953	0.79533476	4.45	0.0136

3.2.1. Input data:

The input data on each page is arranged in two columns (each containing trt, time and resp), from top to bottom and left to right.

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TRT	TIME	RESP	TRT	TIME	RESP	TRT	TIME	RESP
1	1	0.117	1	2	0.174	1	3	.
1	2	0.027	1	3	.			
1	3	.				1	1	0.179
			1	1	0.157	1	2	0.327
1	1	0.037	1	2	0.353	1	3	0.193
1	2	0.079	1	3	.			
1	3	0.084				1	1	0.093
			1	1	0.087	1	2	0.265
1	1	0.087	1	2	0.144	1	3	1.748
1	2	0.092	1	3	0.339			
1	3	.				1	1	0.123
			1	1	1.108	1	2	0.098
1	1	0.170	1	2	0.325	1	3	0.025
1	2	0.236	1	3	.			
1	3	0.062				2	1	0.033
			1	1	0.121	2	2	0.047
1	1	0.012	1	2	0.096	2	3	.
1	2	0.091	1	3	0.134			
1	3	2.017				1	1	0.123
			1	1	0.479	1	2	0.468
2	1	0.005	1	2	0.256	1	3	0.496
2	2	0.034	1	3	0.549			
2	3	.				1	1	0.269
			1	1	0.084	1	2	1.033
1	1	0.207	1	2	0.086	1	3	0.397
1	2	0.133	1	3	0.073			
1	3	.				1	1	0.049
			1	1	0.140	1	2	0.146
1	1	0.109	1	2	0.133	1	3	0.042
1	2	0.126	1	3	.			
1	3	0.146				1	1	0.033
			1	1	0.220	1	2	0.048
2	1	0.061	1	2	0.170	1	3	0.067
2	2	0.175	1	3	0.835			
2	3	0.245				1	1	0.071
			1	1	0.087	1	2	0.073
2	1	0.046	1	2	0.541	1	3	0.051
2	2	0.313	1	3	.			
2	3	2.511				1	1	1.552
			1	1	0.107	1	2	1.365
1	1	0.147	1	2	0.434	1	3	1.156
1	2	0.200	1	3	.			
1	3	0.405				1	1	0.028
			1	1	0.046	1	2	0.091
1	1	0.162	1	2	1.850	1	3	.
1	2	0.218	1	3	0.526			
1	3	0.092				1	1	0.222
			2	1	0.042	1	2	0.292
1	1	0.229	2	2	0.109	1	3	0.613
1	2	0.141	2	3	1.737			
1	3	0.332				2	1	0.120
			1	1	0.039	2	2	0.121
1	1	0.000	1	2	0.062	2	3	0.159

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3.3. Oiled vs non-Oiled: February 2002 to March 2002

The SAS System

General Linear Models Procedure

Class Level Information

Class	Levels	Values
TRT	2	1 2
TIME	3	1 2 3

Number of observations in data set = 30

NOTE: Due to missing values, only 27 observations can be used in this analysis.

Dependent Variable: RESP

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	2	0.23028325	0.11514163	2.72	0.0858
Error	24	1.01411941	0.04225498		
Corrected Total	26	1.24440267			

R-Square		C.V.	Root MSE	RESP Mean	
0.185055		81.28477	0.205560	0.252889	

Source	DF	Type I SS	Mean Square	F Value	Pr > F
TRT	0	0.00000000			
TIME	2	0.23028325	0.11514163	2.72	0.0858
TRT*TIME	0	0.00000000			

3.3.1. Input data:

The input data on each page is arranged in two columns (each containing trt, time and resp), from top to bottom and left to right.

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3.4. Oiled vs non-Oiled: April 2002 to May 2002

The SAS System

General Linear Models Procedure

Class Level Information

Class	Levels	Values
TRT	2	1 2
TIME	3	1 2 3

Number of observations in data set = 195

NOTE: Due to missing values, only 184 observations can be used in this analysis.

Dependent Variable: RESP

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	5	0.29967941	0.05993588	1.81	0.1132
Error	178	5.89595300	0.03312333		
Corrected Total	183	6.19563241			

R-Square		C.V.	Root MSE	RESP Mean	
0.048369		198.4101	0.181998	0.091728	

Source	DF	Type I SS	Mean Square	F Value	Pr > F
TRT	1	0.00088598	0.00088598	0.03	0.8703
TIME	2	0.17094144	0.08547072	2.58	0.0786
TRT*TIME	2	0.12785199	0.06392600	1.93	0.1482

3.4.1. Input data:

The input data on each page is arranged in two columns (each containing trt, time and resp), from top to bottom and left to right.

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TRT	TIME	RESP	TRT	TIME	RESP	TRT	TIME	RESP
1	1	1.125	2	2	0.115	2	3	0.091
1	2	0.246	2	3	0.085			
1	3	0.213				2	1	0.051
			2	1	0.027	2	2	0.104
1	1	0.141	2	2	0.042	2	3	0.061
1	2	0.034	2	3	0.071			
1	3	0.039				1	1	0.080
			2	1	0.028	1	2	0.035
1	1	0.247	2	2	0.075	1	3	.
1	2	0.027	2	3	0.050			
1	3	0.036				2	1	0.049
			2	1	0.016	2	2	0.052
1	1	0.051	2	2	0.044	2	3	.
1	2	0.059	2	3	0.021			
1	3	0.249				2	1	0.091
			2	1	0.017	2	2	0.338
1	1	0.035	2	2	0.017	2	3	0.097
1	2	0.154	2	3	0.019			
1	3	.				2	1	0.065
			2	1	0.031	2	2	0.126
1	1	0.028	2	2	0.059	2	3	0.060
1	2	0.052	2	3	0.025			
1	3	0.045				2	1	0.103
			2	1	0.044	2	2	0.064
1	1	0.020	2	2	0.020	2	3	0.021
1	2	0.037	2	3	.			
1	3	0.057				1	1	0.040
			2	1	0.029	1	2	0.141
1	1	0.049	2	2	0.034	1	3	0.049
1	2	0.088	2	3	0.054			
1	3	0.064				2	1	0.090
			2	1	0.032	2	2	0.021
1	1	0.047	2	2	0.107	2	3	0.061
1	2	0.106	2	3	0.101			
1	3	.				1	1	0.226
			2	1	0.020	1	2	0.052
1	1	0.079	2	2	0.152	1	3	0.070
1	2	0.084	2	3	2.032			
1	3	0.113				1	1	0.057
			2	1	0.030	1	2	0.032
2	1	0.027	2	2	0.077	1	3	0.068
2	2	0.077	2	3	0.067			
2	3	.				1	1	0.026
			2	1	0.063	1	2	0.059
2	1	0.028	2	2	0.040	1	3	0.041
2	2	0.058	2	3	0.029			
2	3	.				1	1	0.030
			2	1	0.028	1	2	0.075
2	1	0.011	2	2	0.038	1	3	0.034
2	2	0.061	2	3	0.278			
2	3	0.023				2	1	0.046
			2	1	0.068	2	2	0.011
2	1	0.020	2	2	0.080	2	3	0.053

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TRT	TIME	RESP
2	1	0.020
2	2	0.003
2	3	0.070
2	1	0.026
2	2	0.027
2	3	0.070
1	1	0.044
1	2	0.038
1	3	0.043
2	1	0.068
2	2	0.118
2	3	.
1	1	0.023
1	2	0.063
1	3	0.122
2	1	0.019
2	2	0.032
2	3	0.061
2	1	0.014
2	2	0.024
2	3	0.070
2	1	0.020
2	2	0.063
2	3	0.074
2	1	0.016
2	2	0.011
2	3	0.366
1	1	0.004
1	2	0.013
1	3	0.086
1	1	0.010
1	2	0.090
1	3	0.092
2	1	0.016
2	2	0.013
2	3	0.048
2	1	0.045
2	2	0.028
2	3	0.043
2	1	0.028
2	2	0.027
2	3	0.093
1	1	0.027
1	2	0.053
1	3	0.222
2	1	0.021
2	2	0.069
2	3	0.045
2	1	0.058
2	2	0.045
2	3	0.060
2	1	0.022
2	2	0.021
2	3	0.342
2	1	0.047
2	2	0.033
2	3	0.043
1	1	0.016
1	2	0.037
1	3	.
1	1	0.015
1	2	0.067
1	3	0.085
1	1	0.083
1	2	0.105
1	3	.
2	1	0.071
2	2	0.122
2	3	0.618
2	1	0.127
2	2	0.514
2	3	.
1	1	0.311
1	2	0.109
1	3	0.219
2	1	0.052
2	2	0.089
2	3	0.360

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3.5. Oiled vs non-Oiled: June 2002 to July 2002

The SAS System

General Linear Models Procedure

Class Level Information

Class	Levels	Values
TRT	2	1 2
TIME	3	1 2 3

Number of observations in data set = 153

NOTE: Due to missing values, only 151 observations can be used in this analysis.

Dependent Variable: RESP

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	5	0.45575727	0.09115145	3.39	0.0063
Error	145	3.89874610	0.02688790		
Corrected Total	150	4.35450338			

R-Square		C.V.	Root MSE	RESP Mean	
0.104663		168.2655	1.163975	0.097450	

Source	DF	Type I SS	Mean Square	F Value	Pr > F
TRT	1	0.02302958	0.02302958	0.86	0.3563
TIME	2	0.37569065	0.18784532	6.99	0.0013
TRT*TIME	2	0.05703704	0.02851852	1.06	0.3489

3.5.1. Input data:

The input data on each page is arranged in two columns (each containing trt, time and resp), from top to bottom and left to right.

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TRT	TIME	RESP	TRT	TIME	RESP	TRT	TIME	RESP
2	1	0.021	2	2	0.239	2	3	0.370
2	2	0.078	2	3	0.051			
2	3	0.042				2	1	0.073
			2	1	0.063	2	2	0.000
2	1	0.037	2	2	0.061	2	3	0.548
2	2	0.065	2	3	0.145			
2	3	0.069				2	1	0.067
			2	1	0.077	2	2	0.000
1	1	0.041	2	2	0.081	2	3	0.976
1	2	0.135	2	3	0.064			
1	3	0.035				2	1	0.112
			2	1	0.112	2	2	0.048
2	1	0.085	2	2	0.172	2	3	0.010
2	2	0.050	2	3	1.394			
2	3	0.053				2	1	0.021
			2	1	0.066	2	2	0.034
2	1	0.081	2	2	0.033	2	3	0.082
2	2	0.049	2	3	0.005			
2	3	0.276				2	1	0.000
			2	1	0.121	2	2	0.019
2	1	0.040	2	2	0.117	2	3	.
2	2	0.022	2	3	0.025			
2	3	0.081				2	1	0.030
			2	1	0.144	2	2	0.023
2	1	0.059	2	2	0.120	2	3	0.046
2	2	0.048	2	3	0.340			
2	3	0.108				2	1	0.034
			1	1	0.065	2	2	0.037
1	1	0.034	1	2	0.046	2	3	0.044
1	2	0.094	1	3	0.011			
1	3	0.088				2	1	0.021
			2	1	0.092	2	2	0.057
1	1	0.063	2	2	0.052	2	3	0.101
1	2	0.107	2	3	0.341			
1	3	0.063				2	1	0.035
			2	1	0.153	2	2	0.029
2	1	0.040	2	2	0.092	2	3	0.042
2	2	0.042	2	3	0.534			
2	3	0.080				2	1	0.027
			2	1	0.130	2	2	0.068
2	1	0.032	2	2	0.049	2	3	0.054
2	2	0.106	2	3	0.975			
2	3	0.016				2	1	0.070
			2	1	0.124	2	2	0.061
2	1	0.019	2	2	0.052	2	3	0.040
2	2	0.069	2	3	0.000			
2	3	0.118				2	1	0.067
			2	1	0.100	2	2	0.082
2	1	0.073	2	2	0.002	2	3	0.042
2	2	0.046	2	3	0.316			
2	3	0.053				2	1	0.062
			2	1	0.182	2	2	0.025
2	1	0.044	2	2	0.000	2	3	0.023

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3.6. Oiled vs non-Oiled: August 2002 to September 2002

The SAS System

General Linear Models Procedure

Class Level Information

Class	Levels	Values
TRT	2	1 2
TIME	3	1 2 3

Number of observations in data set = 183

NOTE: Due to missing values, only 171 observations can be used in this analysis.

Dependent Variable: RESP

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	5	0.14039167	0.02807833	1.31	0.2620
Error	165	3.53534129	0.02142631		
Corrected Total	170	3.67573296			

R-Square		C.V.	Root MSE	RESP Mean	
0.038194		288.6360	0.146377	0.050713	

Source	DF	Type I SS	Mean Square	F Value	Pr > F
TRT	1	0.00367635	0.00367635	0.17	0.6792
TIME	2	0.13648218	0.06824109	3.18	0.0439
TRT*TIME	2	0.00023313	0.00011657	0.01	0.9946

3.6.1. Input data:

The input data on each page is arranged in two columns (each containing trt, time and resp), from top to bottom and left to right.

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TRT	TIME	RESP	TRT	TIME	RESP	TRT	TIME	RESP
2	1	0.010	2	2	0.052	2	3	0.007
2	2	0.023	2	3	0.027			
2	3	.				2	1	0.037
			2	1	0.024	2	2	0.124
1	1	0.024	2	2	0.010	2	3	0.018
1	2	0.022	2	3	0.040			
1	3	.				2	1	0.011
			2	1	0.018	2	2	0.021
2	1	0.000	2	2	0.029	2	3	0.020
2	2	0.072	2	3	0.125			
2	3	.				1	1	0.055
			2	1	0.056	1	2	0.009
2	1	0.000	2	2	0.002	1	3	0.016
2	2	0.035	2	3	0.024			
2	3	0.085				2	1	0.086
			2	1	0.014	2	2	0.002
2	1	0.006	2	2	0.014	2	3	0.014
2	2	0.037	2	3	0.026			
2	3	0.049				1	1	0.029
			1	1	0.034	1	2	0.002
2	1	0.000	1	2	0.007	1	3	0.005
2	2	0.114	1	3	0.929			
2	3	0.090				2	1	0.053
			2	1	0.026	2	2	0.002
2	1	0.005	2	2	0.030	2	3	0.027
2	2	0.021	2	3	0.045			
2	3	0.021				2	1	0.067
			2	1	0.040	2	2	0.025
2	1	0.000	2	2	0.046	2	3	0.006
2	2	0.052	2	3	0.301			
2	3	0.084				2	1	0.028
			2	1	0.044	2	2	0.037
2	1	0.074	2	2	0.079	2	3	0.002
2	2	0.075	2	3	0.108			
2	3	0.118				2	1	0.025
			2	1	0.029	2	2	0.002
1	1	0.102	2	2	0.044	2	3	.
1	2	0.048	2	3	0.043			
1	3	0.034				2	1	0.017
			1	1	0.026	2	2	0.007
2	1	0.075	1	2	0.173	2	3	0.006
2	2	0.046	1	3	0.023			
2	3	1.675				2	1	0.002
			2	1	0.040	2	2	0.027
2	1	0.040	2	2	0.029	2	3	.
2	2	0.011	2	3	0.010			
2	3	0.077				2	1	0.007
			2	1	0.069	2	2	0.000
2	1	0.006	2	2	0.047	2	3	0.032
2	2	0.046	2	3	.			
2	3	0.002				2	1	0.000
			2	1	0.039	2	2	0.008
2	1	0.032	2	2	0.030	2	3	0.034

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TRT	TIME	RESP
2	1	0.000
2	2	0.004
2	3	0.016
2	1	0.021
2	2	0.030
2	3	.
2	1	0.004
2	2	0.030
2	3	.
2	1	0.001
2	2	0.063
2	3	0.013
2	1	0.009
2	2	0.029
2	3	0.016
2	1	0.038
2	2	0.017
2	3	0.008
2	1	0.002
2	2	0.031
2	3	.
1	1	0.000
1	2	0.035
1	3	.
1	1	0.037
1	2	0.159
1	3	0.043
1	1	0.040
1	2	0.053
1	3	0.022
1	1	0.036
1	2	0.035
1	3	0.007
1	1	0.015
1	2	0.028
1	3	0.037
1	1	0.020
1	2	0.024
1	3	0.105
1	1	0.055
1	2	0.084
1	3	.
1	1	0.036
1	2	0.022
1	3	.
2	1	0.036
2	2	0.027
2	3	0.047
2	1	0.015
2	2	0.014
2	3	0.051
2	1	0.042
2	2	0.037
2	3	0.058
2	1	0.023
2	2	0.076
2	3	0.041
1	1	0.023
1	2	0.038
1	3	0.022
1	1	0.021
1	2	0.012
1	3	0.031

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3.7. Oiled vs non-Oiled: October 2002 to November 2002

The SAS System

General Linear Models Procedure

Class Level Information

Class	Levels	Values
TRT	2	1 2
TIME	3	1 2 3

Number of observations in data set = 125

NOTE: Due to missing values, only 116 observations can be used in this analysis.

Dependent Variable: RESP

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	5	0.05686159	0.01137232	1.28	0.2780
Error	110	0.97779227	0.00888902		
Corrected Total	115	1.03465386			

R-Square		C.V.	Root MSE	RESP Mean	
0.054957		229.7619	0.094282	0.041034	

Source	DF	Type I SS	Mean Square	F Value	Pr > F
TRT	1	0.01826012	0.01826012	2.05	0.1546
TIME	2	0.02146111	0.01073055	1.21	0.3030
TRT*TIME	2	0.01714037	0.00857018	0.96	0.3845

3.7.1. Input data:

The input data on each page is arranged in two columns (each containing trt, time and resp), from top to bottom and left to right.

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TRT	TIME	RESP	TRT	TIME	RESP	TRT	TIME	RESP
1	1	0.018	1	2	0.031	2	3	0.010
1	2	0.081	1	3	0.002			
1	3	.				1	1	0.040
			1	1	0.114	1	2	0.024
1	1	0.070	1	2	0.072	1	3	0.019
1	2	0.045	1	3	0.073			
1	3	.				2	1	0.030
			1	1	0.069	2	2	0.020
1	1	0.000	1	2	0.008	2	3	0.040
1	2	0.016	1	3	0.055			
1	3	0.033				1	1	0.032
			2	1	0.025	1	2	0.071
1	1	0.000	2	2	0.002	1	3	0.043
1	2	0.033	2	3	0.030			
1	3	0.020				1	1	0.027
			2	1	0.002	1	2	0.022
1	1	0.020	2	2	0.011	1	3	.
1	2	0.033	2	3	0.007			
1	3	0.038				1	1	0.027
			2	1	0.014	1	2	0.055
2	1	0.005	2	2	0.057	1	3	0.025
2	2	0.013	2	3	0.066			
2	3	.				2	1	0.044
			2	1	0.000	2	2	0.036
1	1	0.023	2	2	0.011	2	3	0.028
1	2	0.035	2	3	0.031			
1	3	.				2	1	0.029
			2	1	0.000	2	2	0.047
2	1	0.043	2	2	0.008	2	3	.
2	2	0.031	2	3	0.012			
2	3	.				2	1	0.070
			2	1	0.000	2	2	0.027
1	1	0.020	2	2	0.013	2	3	0.040
1	2	0.039	2	3	0.050			
1	3	0.008				2	1	0.055
			1	1	0.006	2	2	0.044
1	1	0.021	1	2	0.043	2	3	.
1	2	0.036	1	3	0.027			
1	3					2	1	0.013
			1	1	0.002	2	2	0.017
1	1	0.021	1	2	0.032	2	3	0.020
1	2	0.003	1	3	0.018			
1	3	0.014				2	1	0.026
			1	1	0.050	2	2	0.056
1	1	0.039	1	2	0.013	2	3	.
1	2	0.010	1	3	0.005			
1	3	0.009				2	1	0.049
			1	1	0.118	2	2	0.018
2	1	0.067	1	2	0.108	2	3	0.024
2	2	0.075	1	3	0.035			
2	3	0.002				2	1	0.024
			2	1	0.017	2	2	0.020
1	1	0.015	2	2	0.018	2	3	0.013

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3.8. Oiled vs non-Oiled: December 2002 to January 2003

The SAS System

General Linear Models Procedure

Class Level Information

Class	Levels	Values
TRT	2	1 2
TIME	3	1 2 3

Number of observations in data set = 96

NOTE: Due to missing values, only 89 observations can be used in this analysis.

Dependent Variable: RESP

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	5	0.15036589	0.03007318	1.66	0.1523
Error	83	1.49966461	0.01806825		
Corrected Total	88	1.65003049			

R-Square		C.V.	Root MSE	RESP Mean	
0.091129		185.0459	0.134418	0.072640	

Source	DF	Type I SS	Mean Square	F Value	Pr > F
TRT	1	0.04140574	0.04140574	2.29	0.1339
TIME	2	0.10024210	0.05012105	2.77	0.0682
TRT*TIME	2	0.00871804	0.00435902	0.24	0.7862

3.8.1. Input data:

The input data on each page is arranged in two columns (each containing trt, time and resp), from top to bottom and left to right.

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TRT	TIME	RESP
1	1	0.004
1	2	0.000
1	3	.
1	1	0.000
1	2	0.358
1	3	0.064
1	1	0.000
1	2	0.010
1	3	.
1	1	0.008
1	2	0.055
1	3	0.373
1	1	0.001
1	2	0.013
1	3	0.073
1	1	0.031
1	2	0.222
1	3	.
1	1	0.035
1	2	0.067
1	3	0.013
2	1	0.021
2	2	0.088
2	3	.
1	1	0.022
1	2	0.126
1	3	0.097
1	1	0.117
1	2	0.110
1	3	0.448
1	1	0.004
1	2	0.050
1	3	0.005
1	1	0.000
1	2	0.026
1	3	0.000
1	1	0.037
1	2	0.025
1	3	0.000
1	1	0.059

TRT	TIME	RESP
1	2	0.039
1	3	0.000
1	1	0.020
1	2	0.010
1	3	0.000
2	1	0.003
2	2	0.000
2	3	0.009
2	1	0.002
2	2	0.000
2	3	0.017
2	1	0.009
2	2	0.000
2	3	0.035
1	1	0.002
1	2	0.006
1	3	0.039
1	1	0.004
1	2	0.023
1	3	0.062
1	1	0.005
1	2	0.013
1	3	0.091
1	1	0.018
1	2	0.102
1	3	.
1	1	0.029
1	2	0.074
1	3	.
1	1	0.013
1	2	0.089
1	3	.
1	1	0.083
1	2	0.055
1	3	0.005
1	1	0.045
1	2	0.021
1	3	0.010
1	1	0.024
1	2	0.085

TRT	TIME	RESP
1	3	0.015
1	1	0.034
1	2	0.058
1	3	0.067
1	1	0.046
1	2	0.007
1	3	0.027
1	1	0.085
1	2	0.021
1	3	0.021
1	1	0.134
1	2	0.253
1	3	0.683
1	1	0.092
1	2	0.709
1	3	0.609